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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003, Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 330.00

Complete if Known	
Application Number	09/257,272-Conf. #1980
Filing Date	February 25, 1999
First Named Inventor	Jing-Shan Hu
Examiner Name	R. S. Landsman
Art Unit	1647
Attorney Docket No.	PF112P2D2

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit Card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 08-3425

Deposit Account Name: Human Genome Sciences, Inc.

The Director is authorized to: *(check all that apply)*

☒ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s) or any underpayment of fee(s)

☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	

SUBTOTAL (1) ($) 0.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

			Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		x		=	
Independent Claims		-3** =		x		=	
Multiple Dependent						=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge – late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	330.00
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
Other fee (specify)					

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 330.00

SUBMITTED BY

(Complete *(if applicable)*)

Name (Print/Type)	Melissa J. Pytel	Registration No. (Attorney/Agent)	41,512	Telephone	(301) 610-5764
Signature				Date	May 6, 2004

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Patent Application of: Hu et al.	Docket No.: PF112P2D2
Application No.: 09/257,272	Confirmation No.: 1980
Filed: February 25, 1999	Art Unit: 1647
For: Vascular Endothelial Growth Factor 2	Examiner: R. S. Landsman

APPEAL BRIEF PURSUANT TO 37 CFR § 1.192

Honorable Commissioner of Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal was filed in the above-captioned United States Patent Application on April 5, 2004, appealing the decision of the Patent Office in the Final Office Action (Paper No. 121803, mailed January 5, 2004) in which a final rejection of claims 33-48, 65-96 and 113-272 was issued. Appellants hereby file this Appeal Brief in triplicate, together with the brief filing fee of $330.00 under 37 C.F.R. § 1.17(c).

The Commissioner is hereby authorized to charge any fee deficiency, or credit any overpayment, to Deposit Account No. 08-3425.

TABLE OF CONTENTS

I. Real Party in Interest (37 C.F.R. § 1.192(c)(1))

The real party of interest is Human Genome Sciences, Inc., having a place of business at 14200 Shady Grove Road, Rockville, MD 20850, by virtue of an assignment, of the entire interest from inventors Jing-Shan Hu, Craig A. Rosen and Liang Cao, recorded at Reel 9440 and Frame 0794, filed in parent application Serial No. 08/999,811, which has a filing date of December 24, 1997.

II. Related Appeals and Interferences (37 C.F.R. § 1.192(c)(2))

A Notice of Appeal was filed on March 18, 2004 in United States Application Serial No. 09/107,997 ("the '997 application"). The outstanding rejection in the '997 application is similar to the rejection in the instant appeal. The outstanding rejection in United States Application Serial No. 09/219,442 ("the '442 application") is also similar to the rejection in the instant appeal. As of the date the instant Appeal Brief was filed, Appellants have not filed an Appeal Brief in the '997 application or a Notice of Appeal in the '442 application. However, Appellants wish to notify the Board of these applications in the event that Appellants decide to pursue an Appeal in one or both of the '997 and '442 applications prior to a final determination in the instant appeal.

Appellants' undersigned representative, and, on information and belief, Appellants and the assignee of the entire interest, is/are not aware of any other appeals or interferences related to the above-captioned application that will directly affect or be directly affected by or have a bearing on the Board's decision in the present appeal.

III. Status of the Claims (37 C.F.R. § 1.192(c)(3))

No claim stands allowed. Claims 1-32, 49-64, 97-112 and 273-400 have been canceled. Claims 33-48, 65-96 and 113-272 are pending and stand rejected in the above-identified application and are the subject of this appeal. A copy of the pending claims can be found in **Appendix A.**

IV. Status of Amendments (37 C.F.R. § 1.192(c)(4))

All amendments submitted by Appellants have been entered. No amendments have been filed subsequent to the issuance of the Final Office Action (Paper No. 121803).

V. Summary of the Invention (37 C.F.R. § 1.192(c)(5))

In general, the claimed invention is directed toward isolated Vascular Endothelial Growth Factor 2 (also known as VEGF-2 or VEGF-C). VEGF-2 belongs to the platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) family of growth factors that are involved in regulating the formation of blood vessels and lymphatic vessels within the developing embryo and adult, and in pathological situations such as tumorigenesis. Members of the PDGF/VEGF family all contain a conserved homology domain encompassing eight conserved cysteine residues (See, **Exhibit A**, which includes Figure 3A of the instant application in which the eight conserved cysteine residues are demarcated by boxes). Additionally, members of the PDGF/VEGF family contain a signature motif, PXCVXXXRCXGCCN, which is conserved in VEGF-2 (See, Figure 3A of the instant application in which the signature motif is also demarcated by a box, attached hereto as **Exhibit A**). In addition to the conserved homology domain, the primary translation product of VEGF-2, also referred to as a "proprotein," includes N- and C-terminal polypeptide extensions. The proprotein is proteolytically processed to cleave off the N- and C-terminal polypeptide extensions to yield a mature, secreted form.

A. The Independent Claims

Claims 33, 65, 81, 113, 129, 145, 161, 177, 193, 209, 225, 241, and 257 are independent claims. However, the present appeal centers around the subject matter of independent claims 33, 65, 81, 113, 129 and 145. More particularly, claims 33, 65, 81, 113, 129 and 145 are directed towards "an isolated protein comprising an amino acid sequence at least 90% identical to" either "***a mature form***" or "***a proprotein form***" of a polypeptide of either SEQ ID NO:2 or SEQ ID NO:4, or a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149 or 75698.

The application provides the full length nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequence of VEGF-2 (Figures 1A-1E; attached hereto as **Exhibit B**). SEQ ID NO: 1 was obtained by sequencing the cDNA contained in ATCC Deposit No. 97149 (page 7, line 27 through page 8, line 3). The application also provides the nucleotide (SEQ ID NO:3) and deduced amino acid (SEQ ID NO:4) sequence of a truncated, biologically active form of VEGF-2 (Figures 2A-2D; attached hereto as **Exhibit C**). SEQ ID NO: 3 was obtained by sequencing the cDNA contained in ATCC Deposit Number 75698 (page 8, lines 4-11). As shown in the alignment in the attached **Exhibit D**, SEQ ID NO:2 and SEQ ID NO:4 are identical, except that SEQ ID NO:4 lacks the first 70 amino acids contained in SEQ ID NO:2. Examples of the mature and proprotein forms of VEGF-2 are described in the specification, for example, at page 4, lines 7-10; page 10, lines 10-17 and 28-30; page 21, lines 6-7; and page 24, line 20. The specification also describes proproteins having a "prosequence" or a "presquence," or both, for example, at page 11, lines 15-24. The application also describes mature forms of VEGF-2 resulting from the expression, processing and secretion of the VEGF-2 precursor sequences provided in Figures 1 and 2, and ATCC Deposit Nos. 75698 and 97149, for example, at page 4, lines 19-24 and page 21, line 24 through page 27, line 4. The Working Examples describe experiments in which a mature form of VEGF-2 was naturally and intrinsically generated by recombinant expression from a host cell. See, for example Examples 2-4 and Figures 7-11.

B. The Dependent Claims

The claims that depend from each of the six independent claims discussed above mirror one another. Claims 34-48, 66-80, 82-96, 114-128, 130-144 and 146-160 depend from claims 33, 65, 81, 113, 129, and 145, respectively. Claims 34, 66, 82, 114, 130, and 146 are directed towards a protein that is "at least 95% identical" to the protein recited by the claim from which they depend. Claims 35, 67, 83, 115, 131 and 147 are directed toward a fusion protein comprising the protein recited by the claim from which they depend fused to a heterologous polypeptide. Claims 36, 68, 84, 116, 132, and 148 are directed towards a protein comprising a homodimer. Claims 37, 69, 85, 117, 133 and 149 are directed towards a protein which is glycosylated. Claims 38, 44, 70, 76, 86, 92, 118, 124, 134, 140, 150 and 156 are directed

towards a method of simulating proliferation of endothelial cells or angiogenesis in a patient wherein the patient has wound, tissue or bone damage. Claims 39, 45, 71, 77, 87, 93, 119, 125, 135, 141, 151, and 157 recite a patient having ischemia. Claims 40, 46, 72, 78, 88, 94, 120, 126, 136 142, 152 and 158 recite a patient having coronary artery disease, peripheral vascular disease, or CNS vascular disease. Claims 41, 47, 73, 79, 89, 95, 121, 127, 137, 143, 153 and 159 recite a patient that has had a myocardial infarction. Claims 42, 74, 90, 122, 138 and 154 are directed towards a method that stimulates angiogenesis. Claims 43, 48, 75, 80, 91, 96, 123, 128, 139, 144, 155 and 160 recite a human patient.

The instant application describes variants of VEGF-2, including those having "at least 90%" and "at least 95%" identity, for example, at page 18, line 11 through page 21, line 14. Fusion proteins are described, for example, at page 11, line 11 through page 12, line 2; and page 18, lines 25-30. The application describes VEGF-2 homodimers, for example, at page 41, lines 24-26. Glycosylated forms of VEGF-2 are described, for example, at page 27, lines 8-12. Methods of simulating proliferation of endothelial cells or angiogenesis in a human patient having, for example, wound, tissue or bone damage; ischemia; coronary artery disease; peripheral vascular disease; CNS vascular disease; or a myocardial infarction are described, for example, at page 27, line 13 through page 28, line 24.

VI. Issues on Appeal (37 C.F.R. § 1.192(c)(6))

The issues on appeal are:

A. Whether claims 33-48, 65-96 and 113-272 are properly provisionally rejected under the judicially created doctrine of obviousness-type double patenting over the claimed invention in one or more of the following copending United States Patent Applications: 09/219,442, 09/935,726, 08/465,968, 09/107,997, 10/060,523, 10/127,551 and 10/084,488.

Appellants are not contesting the double patenting rejection. With respect to the double patenting rejection, Appellants agree to file a Terminal Disclaimer in the present application over any claims in the cited copending applications that are or will be allowed/issued prior to allowance of the instant application. In this respect, Appellants note that Application Serial No. 08/465,968 issued as United States Patent No. 6,608,182 on August 19, 2003. Upon resolution

of this appeal, Appellants will file a Terminal Disclaimer over at least United States Patent No. 6,608,182.

Appellants further wish to point out that this is the sole remaining rejection of claims 161-272.

B. Whether claims 33-48, 65-96, and 113-160 are properly rejected under 35 U.S.C. § 112, first paragraph as lacking written description for the terms "mature" and "proprotein." The crux of this issue is whether the amino acid sequence of a "mature" or "proprotein" form of a protein must be provided to comply with the written description requirement of 35 U.S.C. § 112, first paragraph.

VII. Grouping of the Claims (37 C.F.R. § 1.192(c)(7))

A. With respect to the provisional rejection under the judicially created doctrine of obviousness-type double patenting claims 33-48, 65-96, and 113-272 stand or fall together.

B. With respect to the rejection under 35 U.S.C. § 112, first paragraph, claims 33-48, 65-96, and 113-160 stand or fall together.

While Appellants have provided a grouping of the claims for the purposes of this appeal, once a patent is granted, each claim shall be presumed valid independently of the validity of other claims, whether in independent or dependent form, pursuant to 35 U.S.C. §282.

VIII. Argument (37 C.F.R. § 1.192(c)(8))

A. Non-Statutory Double Patenting

i. The Final Rejection

Claims 33-48, 65-96, and 113-272 were rejected under the judicially created doctrine of obviousness-type double patenting over the claimed invention in one or more of the following

copending United States Patent Applications: 09/219,442, 09/935,726, 08/465,968, 09/107,997, 10/060,523, 10/127,551 and 10/084,488 (Paper No. 121803, page 2).

ii. Appellants' Argument

As a preliminary matter, Appellants note that this is the sole remaining rejection of claims 161-272.

With respect to the double patenting rejection, Appellants agree to file a Terminal Disclaimer in the present application over the currently pending claims that are or will be allowed/issued prior to allowance of the instant application. In this respect, Appellants note that Application Serial No. 08/465,968 issued as United States Patent No. 6,608,182 on August 19, 2003. Upon notification of allowable subject matter, Appellants will file a Terminal Disclaimer over at least United States Patent No. 6,608,182.

B. Rejections under 35 U.S.C. § 112, First Paragraph

i. The Final Rejection

Claims 33-48, 65-96 and 113-160 were finally rejected under 35 U.S.C. §112, first paragraph as lacking written description for the terms "proprotein" and "mature" (Paper No. 121803, page 4).

Although the Patent Office agrees that (1) "the term 'mature' and 'proprotein' may very well have accepted meanings to those of ordinary skill in the art," that (2) the mature and proprotein forms "my [sic], in fact, be identifiable," and that (3) "the specification does teach the artisan how to make the 'mature' and 'proprotein' forms of VEGF-2," the claims remain rejected for lack of written description because Appellants "have not provided adequate written description of the amino acid sequence of these forms" (Paper No. 121803, pages 3-4). The Patent Office maintains that adequate written description of the "mature" or "proprotein" forms has not been provided "even if the 'mature portion' is naturally and inherently expressed by a host cell, or is similarly expressed in various cells" (Paper No. 121803, page 4). The Patent Office further maintains that, while the exact protein sequence may not be required for peer-

reviewed journal articles, "the bar for patentability is higher and Applicants must be in possession of what is claimed" (Paper No. 121803, page 4). Consequently, the Patent Office is maintaining that the amino acid sequence of the proprotein and mature forms must be provided in the specification to satisfy the written description requirement.

ii. Summary of Appellants' Argument

Appellants contend that the written description requirement does not require that the amino acid sequence of the mature or proprotein form be provided. The proper standard for determining whether the written description requirement is met is whether one of ordinary skill in the art would have understood the inventor to be in possession of the claimed invention at the time the application was filed. *In re Alton*, 76 F.3d 1168, 1175, 37 U.S.P.Q.2d 1578 (Fed. Cir. 1996).

This is in striking contrast to the position of the Patent Office, which maintains that, "the bar for patentability is higher" than that required for peer-reviewed journal articles (i.e., by one of skill in the art) and that "Applicants must be in possession of what is claimed" (Paper No. 121803, page 4). Appellants emphasize that the Federal Circuit has held that a particular form of disclosure is not necessary to describe claimed subject matter and that the proper standard is whether one of ordinary skill in the art would have understood the inventor to be in possession of the claimed invention. *In re Alton,* 76 F.3d at 1175, 37 U.S.P.Q.2d 1578.

As acknowledged by the Patent Office, the "mature portion" of VEGF-2 is naturally and inherently produced when expressed by a host cell. The Patent Office also agrees that the specification clearly provides one of skill in the art with the information necessary to express VEGF-2 from a host cell. Furthermore, the Patent Office admits that Appellant's usage of the terms "mature" and "proprotein" is consistent with that of the skilled artisan. Finally, the Patent Office acknowledges that the amino acid sequence of a "mature" protein is routine to identify once the protein is obtained and there is no evidence that the "mature" form will be differentially produced.

Therefore, Appellants maintain that it is unnecessary to specifically describe either the mechanics of processing or the resulting processed forms, because the processing is an inherent and predictable event. Consequently, other than the amino acid sequence of the precursor form

(which Appellants provide), no other information is required for one of skill in the art to obtain the mature form, nor is more information required for one to recognize that the Appellants were in possession of the claimed invention. Consequently, Appellants maintain that the amino acid sequence is not necessary for one of skill in the art to recognize that the mature and proprotein forms of VEGF-2 are described by the instant application and HGS Priority Applications Serial Nos. 08/207,550 and 08/465,968 (filed March 8, 1994 and June 6, 1995, respectively) and that the inventors were in possession of the invention as claimed at the time of filing.

iii. Legal Standard for Written Description

The function of the "written description" requirement of 35 U.S.C. 112, first paragraph, is to ensure that Appellants had possession of the claimed subject matter, as of the filing date of application relied on. *In re Wertheim*, 541 F.2d 257, 262, 191 U.S.P.Q. 90 (C.C.P.A. 1976). The inquiry into satisfaction of the written description requirement is factual and depends on the nature of the invention and the amount of knowledge imparted to those of skill in the art by the disclosure. *In re Wertheim*, 541 F.2d at 262, 191 U.S.P.Q. 90. Satisfaction of the "written description" requirement does not require *in haec verba* antecedence in the originally filed application. *In re Lukach*, 442 F.2d 967, 969, 169 U.S.P.Q. 795 (C.C.P.A. 1971). The written description requirement can be satisfied by showing that the disclosed subject matter, when given its 'necessary and only reasonable construction,' inherently (*i.e.*, necessarily) satisfies the limitation in question. *Staehelin v. Secher,* 24 U.S.P.Q.2d 1513, 1520 (BPAI 1992) ("a specification need not describe the exact details for preparing every species within the genus described"). In general, precedent establishes that although the Appellant 'does not have to describe exactly the subject matter claimed, the description must clearly allow persons of skill in the art to recognize that [the Appellant] invented what is claimed.' *In re Gosteli,* 872 F.2d 1008, 1012, 10 U.S.P.Q.2d 1614 (Fed. Cir. 1989).

iv. The Specification and Figures Describe the Subject Matter Defined by Each of the Rejected Claims

a. The Specification Provides Nucleotide and Amino Acid Sequences for VEGF-2

The instant application provides the full length nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequence of VEGF-2, which comprises approximately 419 amino acid residues, in Figures 1A-1E, attached hereto as **Exhibit B**. As described at page 7, line 27 through page 8, line 3, SEQ ID NO: 1 was obtained by sequencing a cDNA clone, which was deposited on May 12, 1995 at the American Type Tissue Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland 20852, and given ATCC Deposit No. 97149. The full-length nucleotide and deduced amino acid sequence of VEGF-2 is also provided in HGS priority application Serial No. 08/465,968 at Figure 1 and page 7.

The nucleotide (SEQ ID NO:3) and deduced amino acid (SEQ ID NO:4) sequence of a truncated, biologically active form of VEGF-2, which comprises approximately 350 amino acid residues, is provided in Figures 2A-2D, attached hereto as **Exhibit C**. As described at page 8, lines 4-11, SEQ ID NO: 3 was obtained by sequencing a cDNA clone, which was deposited on March 4, 1994 at the American Type Tissue Collection (ATCC), 12301 Park Lawn Drive, Rockville, Maryland 20852, and given ATCC Deposit Number 75698. The nucleotide and deduced amino acid sequence of the truncated, biologically active form of VEGF-2 is also provided in HGS priority Application Serial No. 08/207,550 at Figure 1 and page 5.

As shown in the alignment in the attached **Exhibit D**, SEQ ID NO:2 and SEQ ID NO:4 are identical, except that SEQ ID NO:4 lacks the first 70 amino acids contained in SEQ ID NO:2.

b. Proprotein and Mature Forms, and Proteolytically Processing of VEGF-2 are Described in the Specification

Examples of the mature and proprotein forms of VEGF-2 are described in the specification of the instant application and the HGS Priority Applications. For example, the instant specification describes "mature" polypeptides at page 4, lines 7-10; page 10, lines 10-17 and 28-30; page 21, lines 6-7; and page 24, line 20. The specification also describes proproteins having a "prosequence" or a "presequence," or both, for example, at page 11, lines 15-21.

Support for the terms "mature" and "proprotein" also can be found in HGS Priority Application Serial No. 08/207,550, for example, at pages 5-8 and in priority application Serial No. 08/465,968, for example, at pages 7, 9, 12 and 13.

According to the specification, the mature forms do not include additional amino acid residues found in the VEGF-2 precursor, such as a leader or secretory sequence or a presequence (See, the instant application, for example, at page 10, lines 10-17; and page 11, lines 15-24; and HGS Priority Application Serial No. 08/207,550, for example, at page 7; and HGS Priority Application Serial No. 08/465,968, for example, at pages 8-9).

c. *The Specification Describes Mature Forms of VEGF-2 Resulting from Expression of a Precursor Sequence*

The instant specification and the HGS priority applications describe mature forms of VEGF-2 resulting from the expression, processing and secretion of the VEGF-2 precursor sequences provided in Figures 1 and 2, and ATCC Deposit Nos. 75698 and 97149. For example, the instant specification discloses producing mature proteins using recombinant techniques along with various expression systems and host cells for producing the biologically active, mature form (See, for example, page 4, lines 19-24 and page 21, line 24 through page 27, line 4). The use of VEGF-2 precursor sequences for recombinant expression of mature VEGF-2 also finds support in HGS Priority Application Serial No. 08/207,550, for example, at pages 10-15 and in HGS Priority Application Serial No. 08/465,968, for example, at pages 14-20. Thus, recombinant processes, which inherently and intrinsically produce mature forms of VEGF-2 from the structurally defined precursor sequence, are expressly described in both the instant specification and both priority applications.

Furthermore, the Working Examples provided in the instant specification and the priority applications demonstrate that inventors had possession of the claimed invention. Specifically, the Working Examples describe experiments in which a mature form of VEGF-2 was naturally and intrinsically generated by recombinant expression from a host cell. See, for example Examples 2-4 of the instant specification, which describe *in vitro* transcription and translation of a VEGF-2 precursor sequence and cloning and expression of VEGF-2 in two different expression systems. Figures 7-11 of the instant specification, which correspond to the experiments in Examples 2-4, provide photographs of SDS-PAGE gels resolving the mature

VEGF-2 as the predominant form resulting from recombinant expression of the precursor sequence. Similarly, HGS Priority Application Serial No. 08/207,550 describes expression of VEGF-2 by *in vitro* transcription and translation at Example 2 and provides an SDS-PAGE gel at Figure 6. HGS Priority Application Serial No. 08/465,968 also describes experiments in which VEGF-2 was cloned and expressed in two different expression systems at Examples 2 and 3, with the results shown at Figures 3-7.

d. The Specification Describes Other Protein Forms of VEGF-2 and Methods of Treating a Patient using VEGF-2

As a preliminary matter, Appellants wish to point out that the Patent Office has indicated that any previous rejection under 35 U.S.C. §112, first paragraph relating to written description for the "% identity" language has been overcome and thus withdrawn (See, Paper No. 121803, page 3, paragraph 4A). Furthermore, there are no additional written description rejections of the subject matter contained in the remaining dependent claims. However, in the interest of completeness, Appellants provide confirmation that the remaining subject matter of the claims is supported by the specification of the instant application and the priority applications.

The instant application describes variants of VEGF-2, including those having "at least 90%" and "at least 95%" identity, for example, at page 18, line 11 through page 21, line 14. Similarly, variants of VEGF-2 are also described in HGS Priority Application Serial No. 08/207,550 at page 9 and Serial No. 08/465, 968 at pages 12-13. The instant application describes fusion proteins, for example, at page 11, line 11 through page 12, line 2; and page 18, lines 25-30 (See also, HGS Priority Application Serial No. 08/207,550 at pages 9 and 13 and Serial No. 08/465, 968 at pages 10, 12 and 18). The application describes VEGF-2 homodimers, for example, at page 41, lines 24-26 (See also, HGS Priority Application Serial No. 08/207,550 at page 22 and Serial No. 08/465, 968 at pages 24-25). Glycosylated forms of VEGF-2 are described, for example, at page 27, lines 8-12 and in HGS Priority Application Serial No. 08/207,550 at page 16 and Serial No. 08/465, 968 at page 20. Methods of simulating proliferation of endothelial cells or angiogenesis in a human patient having, for example, wound, tissue or bone damage; ischemia; coronary artery disease; peripheral vascular disease; CNS vascular disease; or a myocardial infarction are described, for example, at page 27, line 13

through page 28, line 24 (See also, HGS Priority Application Serial No. 08/207,550 at page 16 and Serial No. 08/465, 968 at pages 20-21).

v. As Acknowledged by the Patent Office, the Mature Form is Inherently Produced by Cells

It is well accepted that by "disclosing in a patent application a device that *inherently* performs a function or has a property, operates according to a theory or has an advantage, a *patent application necessarily discloses* that function, theory or advantage, even though it says nothing explicit concerning it." M.P.E.P. § 2163.07(a).

Although the Patent Office has acknowledged that the "mature" and "proprotein" forms of VEGF-2 are inherently formed in a given expression system and are inherent in the structure of the sequences provided in the application (Paper No. 121803, page 4), in the interest of completeness, Appellants will again provide evidence demonstrating that the "mature" and "proprotein" forms of VEGF-2 are inherently produced and are inherent in the structure of the sequences provided in the application.

Appellants previously submitted a copy of a Declaration by Dr. Stuart Aaronson ("the Aaronson Declaration") during prosecution of the instant application along with a Response filed February 4, 2003. A copy of the Aaronson Declaration is attached hereto as **Exhibit E**. In his declaration, Dr. Aaronson states that the capacity of the VEGF-2 polypeptide to be expressed and proteolytically processed to the mature form of the protein is a natural and intrinsic property of that molecule as a result of the cell's recognition of "signals" present in the amino acid sequence of the precursor form of the polypeptide (See Aaronson Declaration at ¶¶ 13 to 17). Additionally, the Aaronson Declaration demonstrates that both the 350 amino acid form and the 419 amino acid form of VEGF-2 are identically processed to the mature form of VEGF-2, resulting in the secretion of polypeptides of identical molecular weights, as assessed by SDS PAGE. Thus, the Aaronson Declaration provides evidence that one of ordinary skill in the art would recognize that Appellants were in possession of the claimed invention as of the March 8, 1994 filing date of HGS Priority Application Serial No. 08/207,550. *In re Alton*, 76 F.3d at 1175.

Thus, one of skill in the art, armed with the teaching of the instant application and the HGS priority applications would have all the information required to express and isolate a

mature and biologically active form of VEGF-2. Because the Patent Office has agreed that the proprotein and mature forms are inherent in the sequence and inherently produced, as previously indicated, Appellants are prepared to amend the specification, as permitted by M.P.E.P. § 2163.07(a), to explicitly refer, by amino acid sequence, to the inherently produced "mature" and "proprotein" forms of VEGF-2.

vi. As Acknowledged by the Patent Office, the Term "Mature" has an Accepted Meaning to Those of Ordinary Skill in the Art

The Patent Office's position that "the bar for patentability is higher" than what is required by one of skill in the art is clearly erroneous. The legal standard is clear: compliance with written description requirement is adjudged by one skilled in the art.

Claim terms are to be given their broadest reasonable interpretation, consistent with the specification and consistent with the interpretation that one skilled in the art would reach. *In re Cortright,* 165 F.3d 1353, 1358, 49 U.S.P.Q.2d 1464 (Fed. Cir. 1999). There is a "heavy presumption" that terms used in claims have the ordinary meaning that would be attributed to those words by persons skilled in the relevant art. *See CCS Fitness, Inc. v. Brunswick Corp.*, 288 F.3d 1359, 1366, 62 U.S.P.Q.2d 1658 (Fed. Cir. 2002); *K-2 Corp. v. Salomon S.A.*, 191 F.3d 1356, 1362-63, 52 U.S.P.Q.2d 1001 (Fed. Cir. 1999); *Johnson Worldwide Assocs., Inc. v. Zebco Corp.*, 175 F.3d 985, 989, 50 U.S.P.Q.2d 1607 (Fed. Cir. 1999); *Specialty Composites v. Cabot Corp.*, 845 F.2d 981, 986, 6 U.S.P.Q.2d 1601 (Fed. Cir. 1988). Determining the meaning of a technical claim term must not be done in a vacuum, but always in light of the teachings of the prior art, the disclosure as it would be interpreted by one having of skill in the art, and the statements made during prosecution. *In re Moore,* 439 F.2d 1232, 1235 and n.2, 169 U.S.P.Q. 236 (C.C.P.A. 1971). Further, a particular form of disclosure is not necessary to describe claimed subject matter, only a description that clearly allows persons of ordinary skill in the art to recognize that the inventor was in possession of what is claimed. *In re Alton,* 76 F.3d at 1175, 37 U.S.P.Q.2d 1578. Thus, a crucial step in determining the meaning of a technical claim term is to determine the ordinary meaning that would be ascribed by a person skilled in the relevant art.

Technical publications can be used to confirm the skilled artisan's definition of a technical claim term, as well as to show that the patentee intended to apply that definition. *See,*

for example, *Arthur A. Collins, Inc. v. Northern Telecom Ltd.*, 216 F.3d 1042, 1045, 55 U.S.P.Q.2D 1143 (Fed. Cir. 2000) ("[w]hen prior art that sheds light on the meaning of a term is cited by the patentee, it can have particular value as a guide to the proper construction of the term, because it may indicate not only the meaning of the term to persons skilled in the art, but also that *the patentee intended to adopt that meaning*.") (emphasis added). *See also, In re Cortright* at 165 F.3d 1358, 49 U.S.P.Q.2d 1464; *Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1584, 39 U.S.P.Q.2d 1573 (Fed. Cir. 1996).

Appellants have previously submitted technical publications that demonstrate the skilled artisan frequently refers to either the "mature" or "proprotein" forms of a protein without explicitly defining these forms by amino acid sequence. Evidence of this usage is illustrated in **Exhibit F**, where the authors refer to the "mature" and "proprotein" forms of VEGF-2 without defining, or even knowing, the complete amino acid sequence of these forms. See, for example, page 291, second column, second paragraph of Joukov et al., *A Novel Vascular Endothelial Growth Factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) Receptor Tyrosine Kinases*, The Embo Journal, Vol. 15, No. 2 (1996)(**Exhibit F**). It was not until a subsequent publication, attached as **Exhibit G**, did these same authors define the complete amino acid sequence of the "mature" and "proprotein" forms of VEGF-2. *See*, for example, the Abstract, and page 3898, column 2, last paragraph that extends to page 3899, and Figure 10 of Joukov et al., *Proteolytic Processing Regulates Receptor Specificity and Activity of VEGF-C*, The Embo Journal, Vol. 16, No. 13 (1997)(**Exhibit G**). Thus, when referring to the "mature" or "proprotein" forms of a protein, the skilled artisan does not require that the specific amino acid sequence be defined. The PTO is therefore improperly requiring a level of precision in technical claim terms that is inconsistent with how those terms are used in the art.

Appellants have also submitted the Aaronson Declaration, *supra*, which provides evidence that one of skill in the art would recognize that the mature form of VEGF-2 is adequately described by the instant application.

The Patent Office has not provided any evidence to refute either the technical publications or Aaronson Declaration, but rather has agreed with Appellants that the terms "mature" and "proprotein" have art-accepted meanings.

vii. Mature and Proprotein Forms are Defined in the Specification Consistently with the Art's Usage of these Terms.

When technical claim terms are used consistently with the prior art and as understood by the skilled artisan, there is no requirement that the patentee provide a definition of the term in either the specification or the claims. *Johnson Worldwide Associates, Inc. v. Zebco Corp.*, 175 F.3d 985, 990, 50 U.S.P.Q.2d 1607 (Fed. Cir. 1999); *In re Paulsen,* 30 F.3d 1475, 1480, 31 U.S.P.Q.2d 1671 (Fed. Cir. 1994); *Intellicall, Inc. v. Phonometrics, Inc.*, 952 F.2d 1384, 1387-8, 21 U.S.P.Q.2d 1383 (Fed. Cir. 1992) ("Where an inventor chooses to be his own lexicographer and to give terms uncommon meanings, he must set out his uncommon definition in some manner within the patent disclosure").

As outlined in the *section iv*, above, the specification describes "proprotein" and "mature" portions of VEGF-2. The lack of specified amino acid sequences in the specification of the "mature" and "proprotein" forms of VEGF-2 is not a flaw of the specification. Rather, the technical terms "mature" and "proprotein" are being used consistently in the specification with how the terms are routinely used in the art (see, *section vi,* above).

viii. The Patent Office Acknowledges that the Amino Acid Sequences of "Mature" and "Proprotein" Forms are Routine to Identify

The Patent Office acknowledges that it is routine to determine the amino acid sequence of the "mature" and/or the "proprotein" forms of a protein and that "the specification does teach the artisan how to make the 'mature' and 'proprotein' forms of VEGF-2" (Paper No. 121803, page 3).

At the time of filing, it was well known that the full-length proteins of the PDGF/VEGF family undergo proteolytic processing to generate the mature form of the protein. See, for example, **Exhibit G**, page 3906, first column, last sentence. Moreover, at the time of filing it was well known how to N-terminally sequence isolated proteins from cells transfected with a particular gene sequence. Therefore, if necessary, the skilled artisan could have routinely expressed the VEGF-2 sequence disclosed in the specification and N-terminally sequenced the isolated protein, thereby identifying the amino acid sequence of the mature form.

ix. No Evidence that "Mature" Forms will be Differentially Produced

Although the Patent Office has acknowledged that the "mature form" may be "similarly expressed in various cells" (Paper No. 121803, page 4), in the interest of being thorough, Appellants reiterate their argument that there is no evidence that "mature" forms are differentially produced in different cells. Appellants provide herewith a third party publication that demonstrates VEGF-2 is, in fact, processed similarly in different cell types. When the authors of **Exhibit G** expressed VEGF-2 in a variety of host cells, they found that VEGF-2 is proteolytically processed "similarly in different cell types." See, **Exhibit G**, page 3901, second column, last sentence of first complete paragraph.

However, even if the "mature" form of VEGF-2 did process differently depending on the cell type, the claims still would not lack written description. In a case with facts analogous to the present situation, the Federal Circuit recently held that claims directed to proteins produced from a "vertebrate" or "mammalian" host cell satisfied the written description requirement, even though there might be "minor differences" in applying the disclosed methods to any other type of host cell and that those of ordinary skill in the art in 1984 could have "easily" figured out the differences in methodology. *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1331, 65 U.S.P.Q.2d 1385 (Fed. Cir. 2003). Thus, in a case with facts almost identical to the present situation, the Federal Circuit found adequate written description for claims directed to products produced by mammalian or vertebrate host cells. This decision was made on a patent having a filing date in the early 1980s. Clearly, this same analysis should readily apply to the present application. Therefore, even if processing of VEGF-2 is host cell specific, the claims directed to the "mature" and "proprotein" forms of VEGF-2 are fully supported by the specification.

x. Errors in the Patent Office's Rejection (37 C.F.R. § 1.192(c)(8)(i)

Appellants submit that the Patent Office is erroneously requiring that the amino acid sequence of the "mature" or "proprotein" forms of VEGF-2 be provided to comply with the written description requirement of 35 U.S.C. § 112, first paragraph. The Patent Office agrees that (1) "the term 'mature' and 'proprotein' may very well have accepted meanings to those of ordinary skill in the art," that (2) the mature and proprotein forms "my [sic], in fact, be identifiable," that (3)the "mature" and "proprotein" forms may be "naturally and inherently

expressed by a host cell, or ... similarly expressed in various cells," and that (4) "the specification does teach the artisan how to make the 'mature' and 'proprotein' forms of VEGF-2." (Paper No. 121803, pages 3-4).

However, in contravention to the recognized legal standard, the Patent Office is maintaining that the amino acid sequence of the proprotein and mature forms must be provided to comply with the written description requirement of 35 U.S.C. §112, first paragraph because "the bar for patentability is higher [than that of peer-reviewed journal articles] and Applicants must be in possession of what is claimed." See, *In re Alton,* 76 F.3d at 1175, 37 U.S.P.Q.2d 1578 (Fed. Cir. 1996) (A particular form of disclosure is not necessary to describe claimed subject matter).

The legal standard for determining whether a disclosure complies with the written description requirement is whether one of skill in the art would have recognized that the terms find written description in the specification. Appellants submit that the specification as filed is sufficient such that one of skill in the art would understand that Appellants were in possession of the claimed invention. Furthermore, as indicated previously, Appellants are willing to amend the specification to explicitly refer, by amino acid sequence, to the inherently produced "mature" and "proprotein" forms of VEGF-2.

IX. Conclusion

It is unnecessary for Appellants to explicitly define by amino acid sequence, the beginning and end of the proprotein and mature forms of VEGF-2. The instant specification and the specification of the priority applications contain sufficient information for one of ordinary skill in the art to recognize that the Appellants were in possession of the invention as claimed.

In view of the entire record, Appellants request that the rejection under 35 U.S.C. § 112, first paragraph be reversed.

Dated: May 6, 2004

Respectfully submitted,

By _____

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APPENDIX A

Pending Claims

1-32. (Canceled)

33. An isolated protein comprising an amino acid sequence at least 90% identical to a mature form of a polypeptide of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

34. The isolated protein of claim 33, wherein said amino acid sequence is at least 95% identical to a mature form of a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

35. A fusion protein comprising the isolated protein of Claim 33 fused to a heterologous polypeptide.

36. The isolated protein of Claim 33 comprising a homodimer.

37. The isolated protein of Claim 33 which is glycosylated.

38. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 33, wherein the patient has a wound, tissue, or bone damage.

39. The method of claim 38, wherein said patient has ischemia.

40. The method of claim 38, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

41. The method of claim 38, wherein said patient has had a myocardial infarction.

42. The method of claim 38, wherein the method stimulates angiogenesis.

43. The method of claim 38, wherein the patient is a human.

44. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 33, wherein the patient has a wound, tissue, or bone damage.

45. The method of claim 44, wherein said patient has ischemia.

46. The method of claim 44, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

47. The method of claim 44, wherein said patient has had a myocardial infarction.

48. The method of claim 44, wherein said patient is a human.

49-64. (Canceled)

65. An isolated protein comprising an amino acid sequence at least 90% identical to a proprotein form of a polypeptide of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

66. The isolated protein of claim 65, wherein said amino acid sequence is at least 95% identical to a proprotein form of a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

67. A fusion protein comprising the isolated protein of Claim 65 fused to a heterologous polypeptide.

68. The isolated protein of Claim 65 comprising a homodimer.

69. The isolated protein of Claim 65 which is glycosylated.

70. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 65, wherein the patient has a wound, tissue, or bone damage.

71. The method of claim 70, wherein said patient has ischemia.

72. The method of claim 70, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

73. The method of claim 70, wherein said patient has had a myocardial infarction.

74. The method of claim 70, wherein the method stimulates angiogenesis.

75. The method of claim 70, wherein the patient is a human.

76. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 65, wherein the patient has a wound, tissue, or bone damage.

77. The method of claim 76, wherein said patient has ischemia.

78. The method of claim 76, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

79. The method of claim 76, wherein said patient has had a myocardial infarction.

80. The method of claim 76, wherein said patient is a human.

81. An isolated protein comprising an amino acid sequence at least 90% identical to a proprotein form of a polypeptide of SEQ ID NO:4, wherein said isolated protein proliferates endothelial cells.

82. The isolated protein of claim 81, wherein said amino acid sequence is at least 95% identical to a proprotein form of a polypeptide comprising the amino acid sequence of SEQ ID NO:4.

83. A fusion protein comprising the isolated protein of Claim 81 fused to a heterologous polypeptide.

84. The isolated protein of Claim 81 comprising a homodimer.

85. The isolated protein of Claim 81 which is glycosylated.

86. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 81, wherein the patient has a wound, tissue, or bone damage.

87. The method of claim 86, wherein said patient has ischemia.

88. The method of claim 86, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

89. The method of claim 86, wherein said patient has had a myocardial infarction.

90. The method of claim 86, wherein the method stimulates angiogenesis.

91. The method of claim 86, wherein the patient is a human.

92. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 81, wherein the patient has a wound, tissue, or bone damage.

93. The method of claim 92, wherein said patient has ischemia.

94. The method of claim 92, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

95. The method of claim 92, wherein said patient has had a myocardial infarction.

96. The method of claim 92, wherein said patient is a human.

97-112. (Canceled)

113. An isolated protein comprising an amino acid sequence that is at least 90% identical to a mature form of a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149, wherein said isolated protein proliferates endothelial cells.

114. The isolated protein of claim 113, wherein said amino acid sequence is at least 95% identical to a mature form of a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149.

115. A fusion protein comprising the isolated protein of Claim 113 fused to a heterologous polypeptide.

116. The isolated protein of Claim 113 comprising a homodimer.

117. The isolated protein of Claim 113 which is glycosylated.

118. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 113, wherein the patient has a wound, tissue, or bone damage.

119. The method of claim 118, wherein said patient has ischemia.

120. The method of claim 118, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

121. The method of claim 118, wherein said patient has had a myocardial infarction.

122. The method of claim 118, wherein the method stimulates angiogenesis.

123. The method of claim 118, wherein the patient is a human.

124. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 113, wherein the patient has a wound, tissue, or bone damage.

125. The method of claim 124, wherein said patient has ischemia.

126. The method of claim 124, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

127. The method of claim 124, wherein said patient has had a myocardial infarction.

128. The method of claim 124, wherein said patient is a human.

129. An isolated protein comprising an amino acid sequence that is at least 90% identical to a proprotein form of a polypeptide encoded by the cDNA contained in ATCC Deposit No. 75698, wherein said isolated protein proliferates endothelial cells.

130. The isolated protein of claim 129, wherein said amino acid sequence is at least 95% identical to a proprotein form of a polypeptide encoded by the cDNA contained in ATCC Deposit No. 75698.

131. A fusion protein comprising the isolated protein of Claim 129 fused to a heterologous polypeptide.

132. The isolated protein of Claim 129 comprising a homodimer.

133. The isolated protein of Claim 129 which is glycosylated.

134. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 129, wherein the patient has a wound, tissue, or bone damage.

135. The method of claim 134, wherein said patient has ischemia.

136. The method of claim 134, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

137. The method of claim 134, wherein said patient has had a myocardial infarction.

138. The method of claim 134, wherein the method stimulates angiogenesis.

139. The method of claim 134, wherein the patient is a human.

140. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 129, wherein the patient has a wound, tissue, or bone damage.

141. The method of claim 140, wherein said patient has ischemia.

142. The method of claim 140, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

143. The method of claim 140, wherein said patient has had a myocardial infarction.

144. The method of claim 140, wherein said patient is a human.

145. An isolated protein comprising an amino acid sequence that is at least 90% identical to a proprotein form of a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149, wherein said isolated protein proliferates endothelial cells.

146. The isolated protein of claim 145, wherein said amino acid sequence is at least 95% identical to a proprotein form of a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149.

147. A fusion protein comprising the isolated protein of Claim 145 fused to a heterologous polypeptide.

148. The isolated protein of Claim 145 comprising a homodimer.

149. The isolated protein of Claim 145 which is glycosylated.

150. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 145, wherein the patient has a wound, tissue, or bone damage.

151. The method of claim 150, wherein said patient has ischemia.

152. The method of claim 150, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

153. The method of claim 150, wherein said patient has had a myocardial infarction.

154. The method of claim 150, wherein the method stimulates angiogenesis.

155. The method of claim 150, wherein the patient is a human.

156. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 145, wherein the patient has a wound, tissue, or bone damage.

157. The method of claim 156, wherein said patient has ischemia.

158. The method of claim 156, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

159. The method of claim 156, wherein said patient has had a myocardial infarction.

160. The method of claim 156, wherein said patient is a human.

161. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide encoded by the cDNA contained in ATCC Deposit No. 75698, wherein said isolated protein proliferates endothelial cells.

162. The isolated protein of claim 161, wherein said amino acid sequence is at least 95% identical to a polypeptide encoded by the cDNA contained in ATCC Deposit No. 75698.

163. A fusion protein comprising the isolated protein of Claim 161 fused to a heterologous polypeptide.

164. The isolated protein of Claim 161 comprising a homodimer.

165. The isolated protein of Claim 161 which is glycosylated.

166. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 161, wherein the patient has a wound, tissue, or bone damage.

167. The method of claim 166, wherein said patient has ischemia.

168. The method of claim 166, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

169. The method of claim 166, wherein said patient has had a myocardial infarction.

170. The method of claim 166, wherein the method stimulates angiogenesis.

171. The method of claim 166, wherein the patient is a human.

172. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 161, wherein the patient has a wound, tissue, or bone damage.

173. The method of claim 172, wherein said patient has ischemia.

174. The method of claim 172, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

175. The method of claim 172, wherein said patient has had a myocardial infarction.

176. The method of claim 172, wherein said patient is a human.

177. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149, wherein said isolated protein proliferates endothelial cells.

178. The isolated protein of claim 177, wherein said amino acid sequence is at least 95% identical to a polypeptide encoded by the cDNA contained in ATCC Deposit No. 97149.

179. A fusion protein comprising the isolated protein of Claim 177 fused to a heterologous polypeptide.

180. The isolated protein of Claim 177 comprising a homodimer.

181. The isolated protein of Claim 177 which is glycosylated.

182. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 177, wherein the patient has a wound, tissue, or bone damage.

183. The method of claim 182, wherein said patient has ischemia.

184. The method of claim 182, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

185. The method of claim 182, wherein said patient has had a myocardial infarction.

186. The method of claim 182, wherein the method stimulates angiogenesis.

187. The method of claim 182, wherein the patient is a human.

188. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 177, wherein the patient has a wound, tissue, or bone damage.

189. The method of claim 188, wherein said patient has ischemia.

190. The method of claim 188, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

191. The method of claim 188, wherein said patient has had a myocardial infarction.

192. The method of claim 188, wherein said patient is a human.

193. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide comprising amino acids 71 to 396 of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

194. The isolated protein of claim 193, wherein said amino acid sequence is at least 95% identical to a polypeptide comprising amino acids 71 to 396 of SEQ ID NO:2.

195. A fusion protein comprising the isolated protein of Claim 193 fused to a heterologous polypeptide.

196. The isolated protein of Claim 193 comprising a homodimer.

197. The isolated protein of Claim 193 which is glycosylated.

198. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 193, wherein the patient has a wound, tissue, or bone damage.

199. The method of claim 198, wherein said patient has ischemia.

200. The method of claim 198, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

201. The method of claim 198, wherein said patient has had a myocardial infarction.

202. The method of claim 198, wherein the method stimulates angiogenesis.

203. The method of claim 198, wherein the patient is a human.

204. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 193, wherein the patient has a wound, tissue, or bone damage.

205. The method of claim 204, wherein said patient has ischemia.

206. The method of claim 204, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

207. The method of claim 204, wherein said patient has had a myocardial infarction.

208. The method of claim 204, wherein said patient is a human.

209. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide comprising amino acids 47 to 396 of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

210. The isolated protein of claim 209, wherein said amino acid sequence is at least 95% identical to a polypeptide comprising amino acids 47 to 396 of SEQ ID NO:2.

211. A fusion protein comprising the isolated protein of Claim 209 fused to a heterologous polypeptide.

212. The isolated protein of Claim 209 comprising a homodimer.

213. The isolated protein of Claim 209 which is glycosylated.

214. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 209, wherein the patient has a wound, tissue, or bone damage.

215. The method of claim 214, wherein said patient has ischemia.

216. The method of claim 214, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

217. The method of claim 214, wherein said patient has had a myocardial infarction.

218. The method of claim 214, wherein the method stimulates angiogenesis.

219. The method of claim 214, wherein the patient is a human.

220. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 209, wherein the patient has a wound, tissue, or bone damage.

221. The method of claim 220, wherein said patient has ischemia.

222. The method of claim 220, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

223. The method of claim 220, wherein said patient has had a myocardial infarction.

224. The method of claim 220, wherein said patient is a human.

225. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide comprising amino acids 24 to 396 of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

226. The isolated protein of claim 225, wherein said amino acid sequence is at least 95% identical to a polypeptide comprising amino acids 24 to 396 of SEQ ID NO:2.

227. A fusion protein comprising the isolated protein of Claim 225 fused to a heterologous polypeptide.

228. The isolated protein of Claim 225 comprising a homodimer.

229. The isolated protein of Claim 225 which is glycosylated.

230. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 225, wherein the patient has a wound, tissue, or bone damage.

231. The method of claim 230, wherein said patient has ischemia.

232. The method of claim 230, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

233. The method of claim 230, wherein said patient has had a myocardial infarction.

234. The method of claim 230, wherein the method stimulates angiogenesis.

235. The method of claim 230, wherein the patient is a human.

236. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 225, wherein the patient has a wound, tissue, or bone damage.

237. The method of claim 236, wherein said patient has ischemia.

238. The method of claim 236, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

239. The method of claim 236, wherein said patient has had a myocardial infarction.

240. The method of claim 236, wherein said patient is a human.

241. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide comprising amino acids 1 to 396 of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

242. The isolated protein of claim 241, wherein said amino acid sequence is at least 95% identical to a polypeptide comprising amino acids 1 to 396 of SEQ ID NO:2.

243. A fusion protein comprising the isolated protein of Claim 241 fused to a heterologous polypeptide.

244. The isolated protein of Claim 241 comprising a homodimer.

245. The isolated protein of Claim 241 which is glycosylated.

246. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 241, wherein the patient has a wound, tissue, or bone damage.

247. The method of claim 246, wherein said patient has ischemia.

248. The method of claim 246, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

249. The method of claim 246, wherein said patient has had a myocardial infarction.

250. The method of claim 246, wherein the method stimulates angiogenesis.

251. The method of claim 246, wherein the patient is a human.

252. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 241, wherein the patient has a wound, tissue, or bone damage.

253. The method of claim 252, wherein said patient has ischemia.

254. The method of claim 252, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

255. The method of claim 252, wherein said patient has had a myocardial infarction.

256. The method of claim 252, wherein said patient is a human.

257. An isolated protein comprising an amino acid sequence that is at least 90% identical to a polypeptide comprising amino acids -23 to 396 of SEQ ID NO:2, wherein said isolated protein proliferates endothelial cells.

258. The isolated protein of claim 257, wherein said amino acid sequence is at least 95% identical a polypeptide comprising amino acids –23 to 396 of SEQ ID NO:2.

259. A fusion protein comprising the isolated protein of Claim 257 fused to a heterologous polypeptide.

260. The isolated protein of Claim 257 comprising a homodimer.

261. The isolated protein of Claim 257 which is glycosylated.

262. A method of stimulating proliferation of endothelial cells in a patient comprising administering to the patient the isolated protein of claim 257, wherein the patient has a wound, tissue, or bone damage.

263. The method of claim 262, wherein said patient has ischemia.

264. The method of claim 262, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

265. The method of claim 262, wherein said patient has had a myocardial infarction.

266. The method of claim 262, wherein the method stimulates angiogenesis.

267. The method of claim 262, wherein the patient is a human.

268. A method of stimulating angiogenesis in a patient comprising administering to the patient the isolated protein of claim 257, wherein the patient has a wound, tissue, or bone damage.

269. The method of claim 268, wherein said patient has ischemia.

270. The method of claim 268, wherein said patient has coronary artery disease, peripheral vascular disease, or CNS vascular disease.

271. The method of claim 268, wherein said patient has had a myocardial infarction.

272. The method of claim 268, wherein said patient is a human.

273-400. (Canceled)

APPENDIX B

Exhibit A: Figure 3A-B

Exhibit B: Figures 1A-1E

Exhibit C: Figures 2A-2D

Exhibit D: Declaration of Stuart Aaronson

Exhibit E: Alignment of Full-Length and Truncated Forms of VEGF-2

Exhibit F: Joukov et al., "A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases." EMBO, 15(2):290-298 (1996)

Exhibit G: Joukov et al., "Proteolytic processing regulates receptor specificity and activity of VEGF-C." EMBO, 16 (13):3898-3911 (1997)

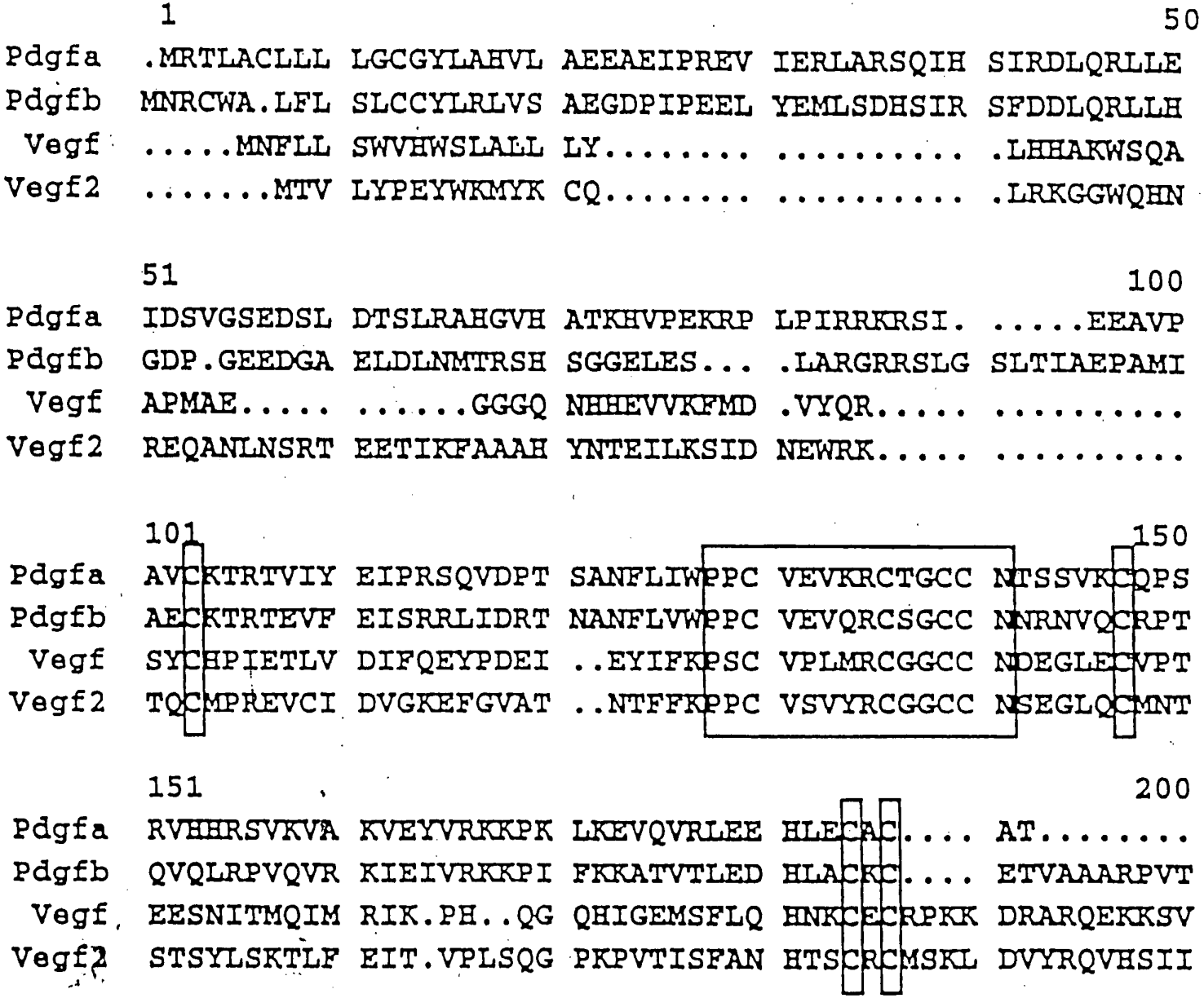

1 50
Pdgfa .MRTLACLLL LGCGYLAHVL AEEAEIPREV IERLARSQIH SIRDLQRLLE
Pdgfb MNRCWA.LFL SLCCYLRLVS AEGDPIPEEL YEMLSDHSIR SFDDLQRLLH
Vegf .....MNFLL SWVHWSLALL LY..... ..... .LHHAKWSQA
Vegf2 .....MTV LYPEYWKMYK CQ..... ..... .LRKGGWQHN
51 100
Pdgfa IDSVGSEDSL DTSLRAHGVH ATKHVPEKRP LPIRRKRSI. .....EEAVP
Pdgfb GDP.GEEDGA ELDLNMTRSH SGGELES... .LARGRRSLG SLTIAEPAMI
Vegf APMAE..... .....GGGQ NHHEVVKFMD .VYQR..... .....
Vegf2 REQANLNSRT EETIKFAAAH YNTEILKSID NEWRK..... .....
101 150
Pdgfa AVCKTRTVIY EIPRSQVDPT SANFLIWPPC VEVKRCTGCC NTSSVKCQPS
Pdgfb AECKTRTEVF EISRRLIDRT NANFLVWPPC VEVQRCSGCC NNRNVQCRPT
Vegf SYCHPIETLV DIFQEYPDEI ..EYIFKPSC VPLMRCGGCC NDEGLECVPT
Vegf2 TQCMPREVCI DVGKEFGVAT ..NTFFKPPC VSVYRCGGCC NSEGLQCMNT
151 200
Pdgfa RVHHRSVKVA KVEYVRKKPK LKEVQVRLEE HLECAC.... AT.....
Pdgfb QVQLRPVQVR KIEIVRKKPI FKKATVTLED HLACKC.... ETVAAARPVT
Vegf EESNITMQIM RIK.PH..QG QHIGEMSFLQ HNKCECRPKK DRARQEKKSV
Vegf2 STSYLSKTLF EIT.VPLSQG PKPVTISFAN HTSCRCMSKL DVYRQVHSII

FIG. 3A

```
        201                                                        250
Pdgfa   ....TSLNPD YREEDTDVR. .......... .......... ..........
Pdgfb   RSPGGSQEQR AKTPQTRVTI RTVRVRRPPK GKHRKFKHTH DKTALKETLG
 Vegf   RGK....... .GKGQKRKRK KSRYKSWSVY VGARCCLMPW SLPGPHP...
Vegf2   RRSLPATLPQ CQAANKTCPT NYMWNNHICR CLAQEDFMFS SDAGDDSTDG

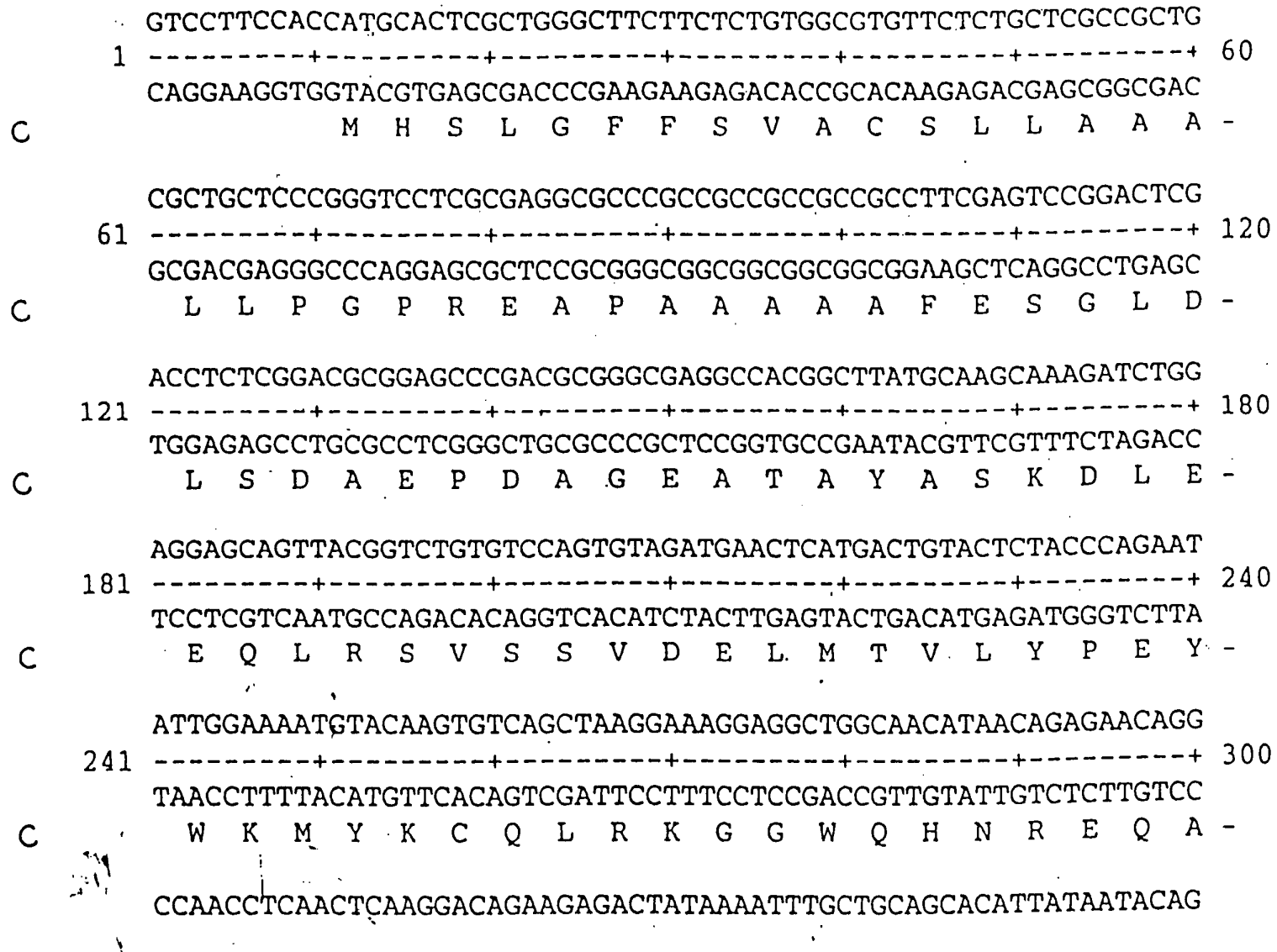
        251                                                        300
Pdgfa   .......... .......... .......... .......... ..........
Pdgfb   A......... .......... .......... .......... ..........
 Vegf   ....CGP... .......... .......CSE RRKHLFVQDP QTCKCSCKNT
Vegf2   FHDICGPNKE LDEETCQCVC RAGLRPASCG PHKEL...DR NSCQCVCKNK

        301                                                        350
Pdgfa   .......... .......... .......... .......... ..........
Pdgfb   .......... .......... .......... .......... ..........
 Vegf   ..DSRCKARQ LELNERTCRC DKPRR..... .......... ..........
Vegf2   LFPSQCGANR .EFDENTCQC VCKRTCPRNQ PLNPGKCACE CTESPQKCLL

        351                                                    398
Pdgfa   .........  .......... .......... .......... ........
Pdgfb   .......... .......... .......... .......... ........
 Vegf   .......... .......... .......... .......... ........
Vegf2   KGKKFHHQTC SCYRRPCTNR QKACEPGFSY SEEVCRCVPS YWQRPQMS
```

FIG. 3B

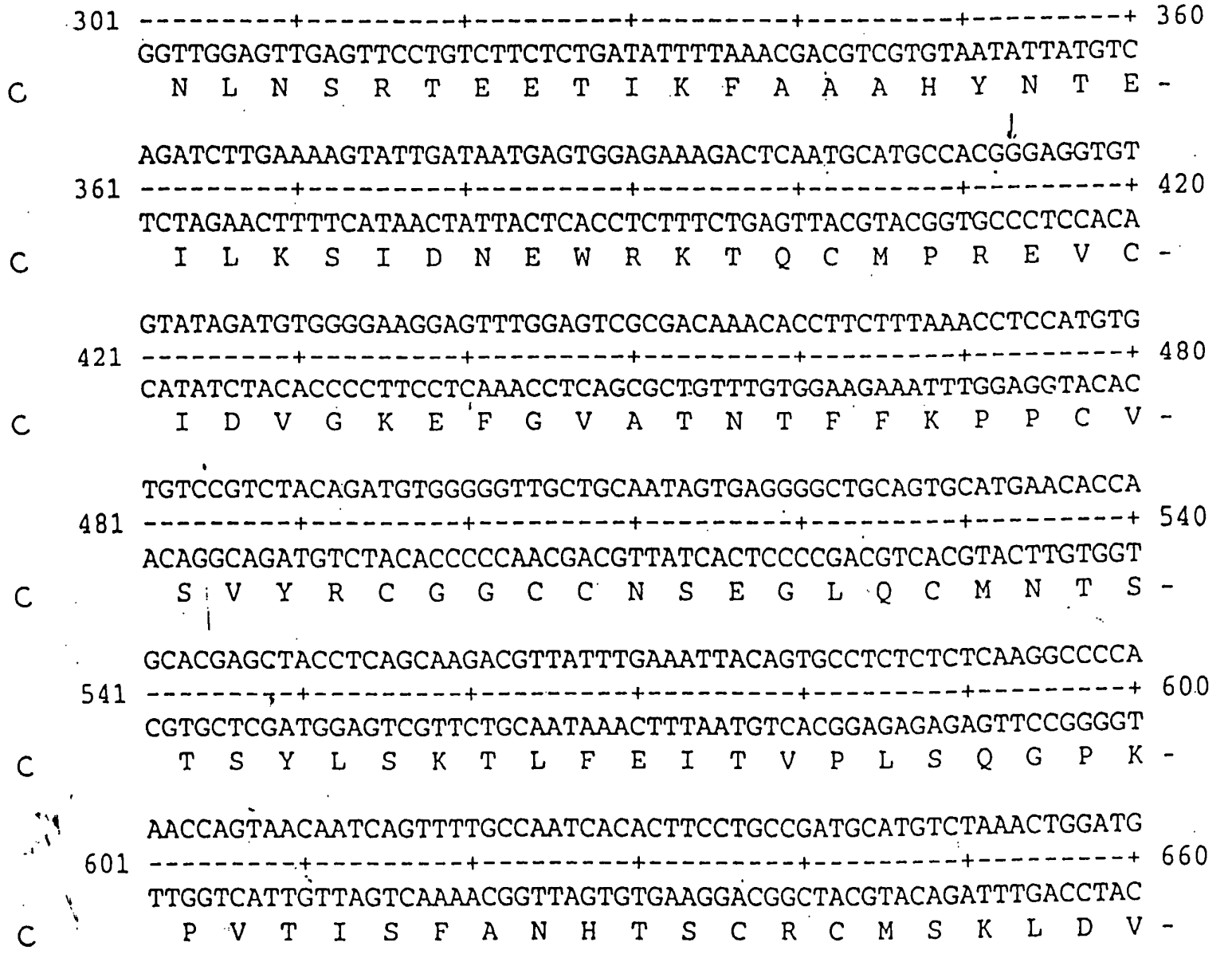
FIG. 1A



MATCH WITH FIG. 1B

MATCH WITH FIG. 1A

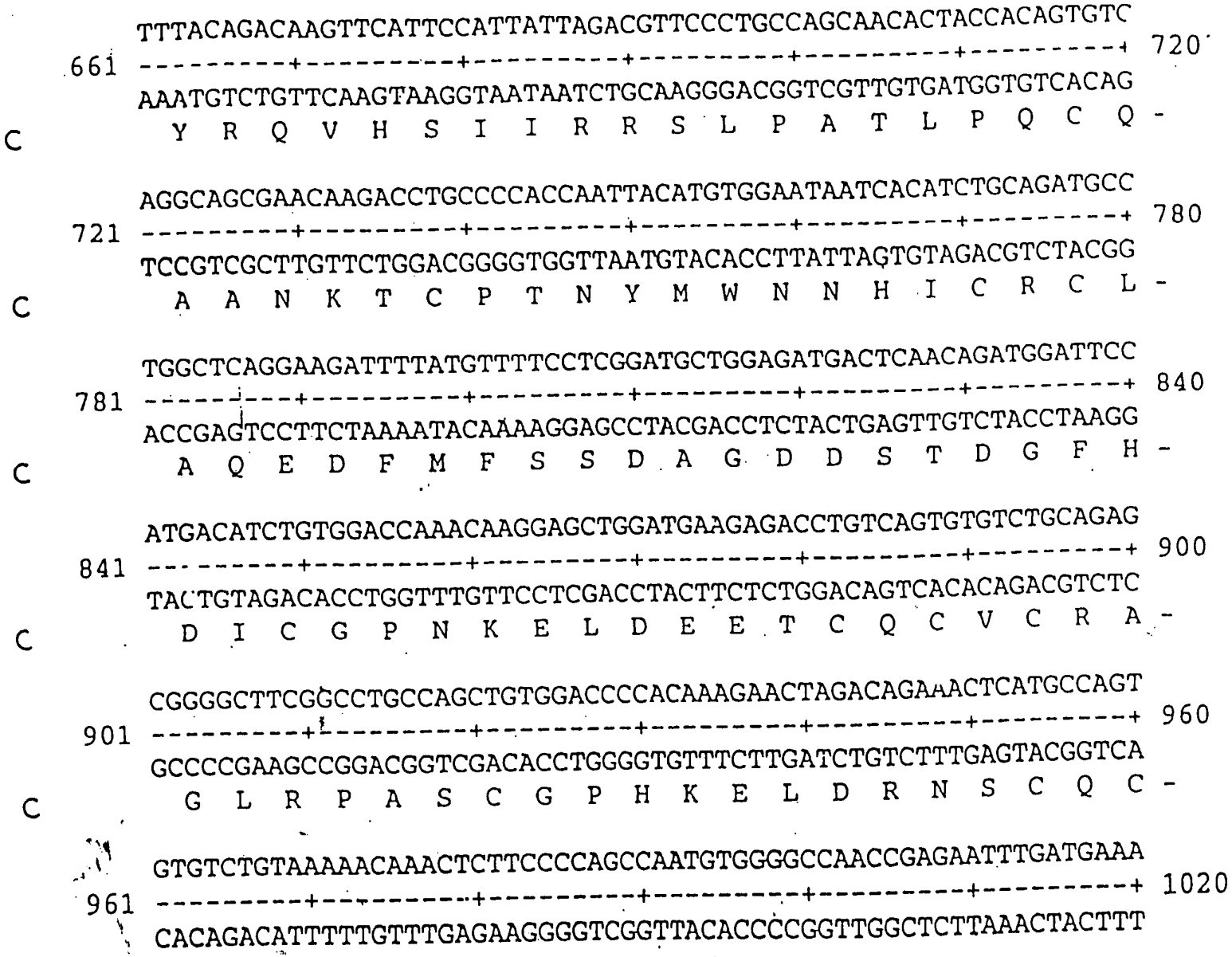
FIG. 1B



MATCH WITH FIG. 1C

MATCH WITH FIG. 1B

FIG. 1C



MATCH WITH FIG. 1D

FIG. 1D

MATCH WITH FIG. 1C

```
C       V  C  K  N  K  L  F  P  S  Q  C  G  A  N  R  E  F  D  E  N -

        ACACATGCCAGTGTGTATGTAAAAGAACCTGCCCCAGAAATCAACCCCTAAATCCTGGAA
1021    ---------+---------+---------+---------+---------+---------+ 1080
        TGTGTACGGTCACACATACATTTTCTTGGACGGGGTCTTTAGTTGGGGATTTAGGACCTT
C         T  C  Q  C  V  C  K  R  T  C  P  R  N  Q  P  L  N  P  G  K -

        AATGTGCCTGTGAATGTACAGAAAGTCCACAGAAATGCTTGTTAAAAGGAAAGAAGTTCC
1081    ---------+---------+---------+---------+---------+---------+ 1140
        TTACACGGACACTTACATGTCTTTCAGGTGTCTTTACGAACAATTTTCCTTTCTTCAAGG
C         C  A  C  E  C  T  E  S  P  Q  K  C  L  L  K  G  K  K  F  H -

        ACCACCAAACATGCAGCTGTTACAGACGGCCATGTACGAACCGCCAGAAGGCTTGTGAGC
1141    ---------+---------+---------+---------+---------+---------+ 1200
        TGGTGGTTTGTACGTCGACAATGTCTGCCGGTACATGCTTGGCGGTCTTCCGAACACTCG
C         H  Q  T  C  S  C  Y  R  R  P  C  T  N  R  Q  K  A  C  E  P -

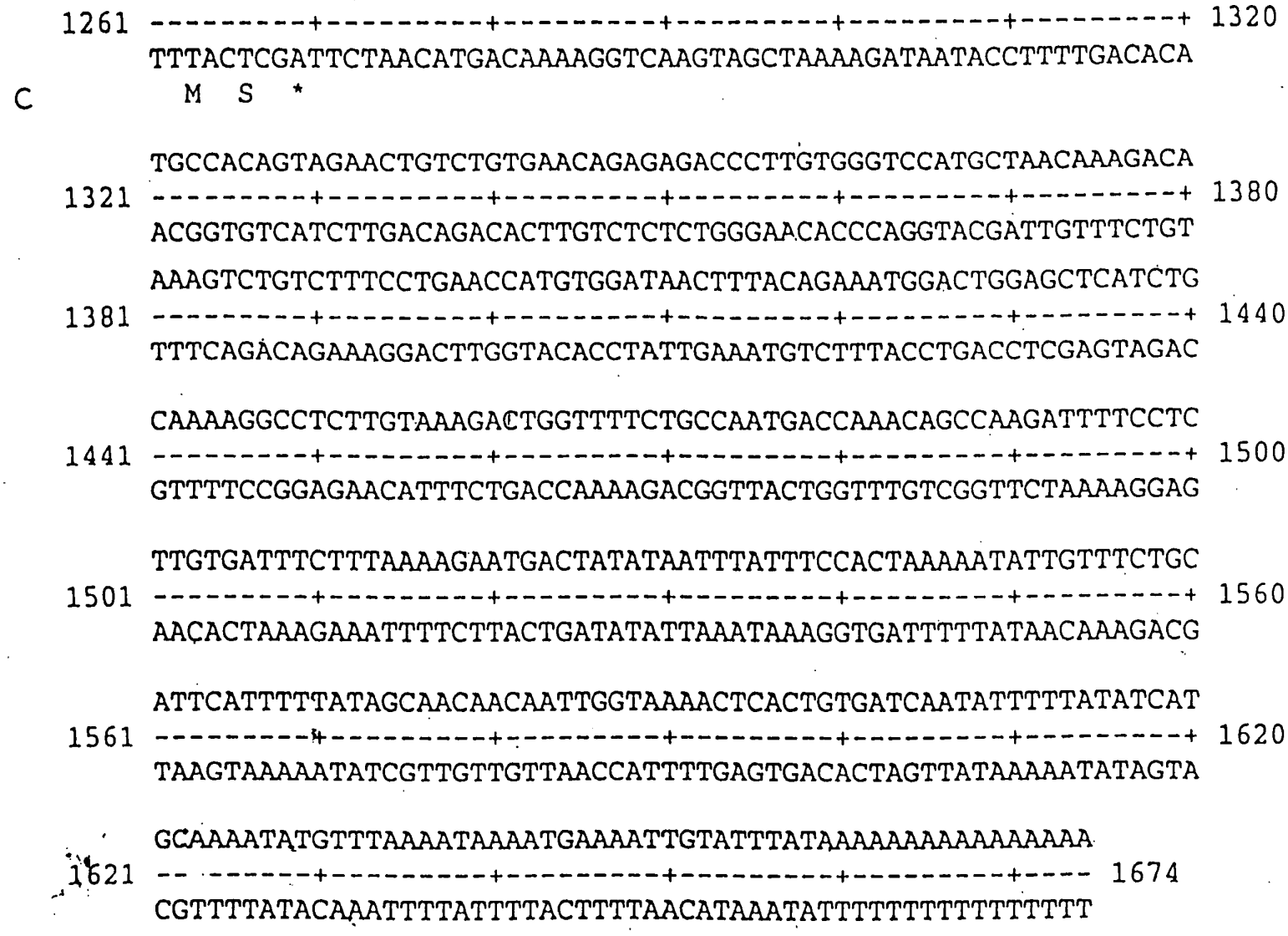
        CAGGATTTTCATATAGTGAAGAAGTGTGTCGTTGTGTCCCTTCATATTGGCAAAGACCAC
1201    ---------+---------+---------+---------+---------+---------+ 1260
        GTCCTAAAAGTATATCACTTCTTCACACAGCAACACAGGGAAGTATAACCGTTTCTGGTG
C         G  F  S  Y  S  E  E  V  C  R  C  V  P  S  Y  W  Q  R  P  Q -

        AAATGAGCTAAGATTGTACTGTTTTCCAGTTCATCGATTTTCTATTATGGAAAACTGTGT
```

MATCH WITH FIG. 1E

MATCH WITH FIG. 1D

FIG. 1E



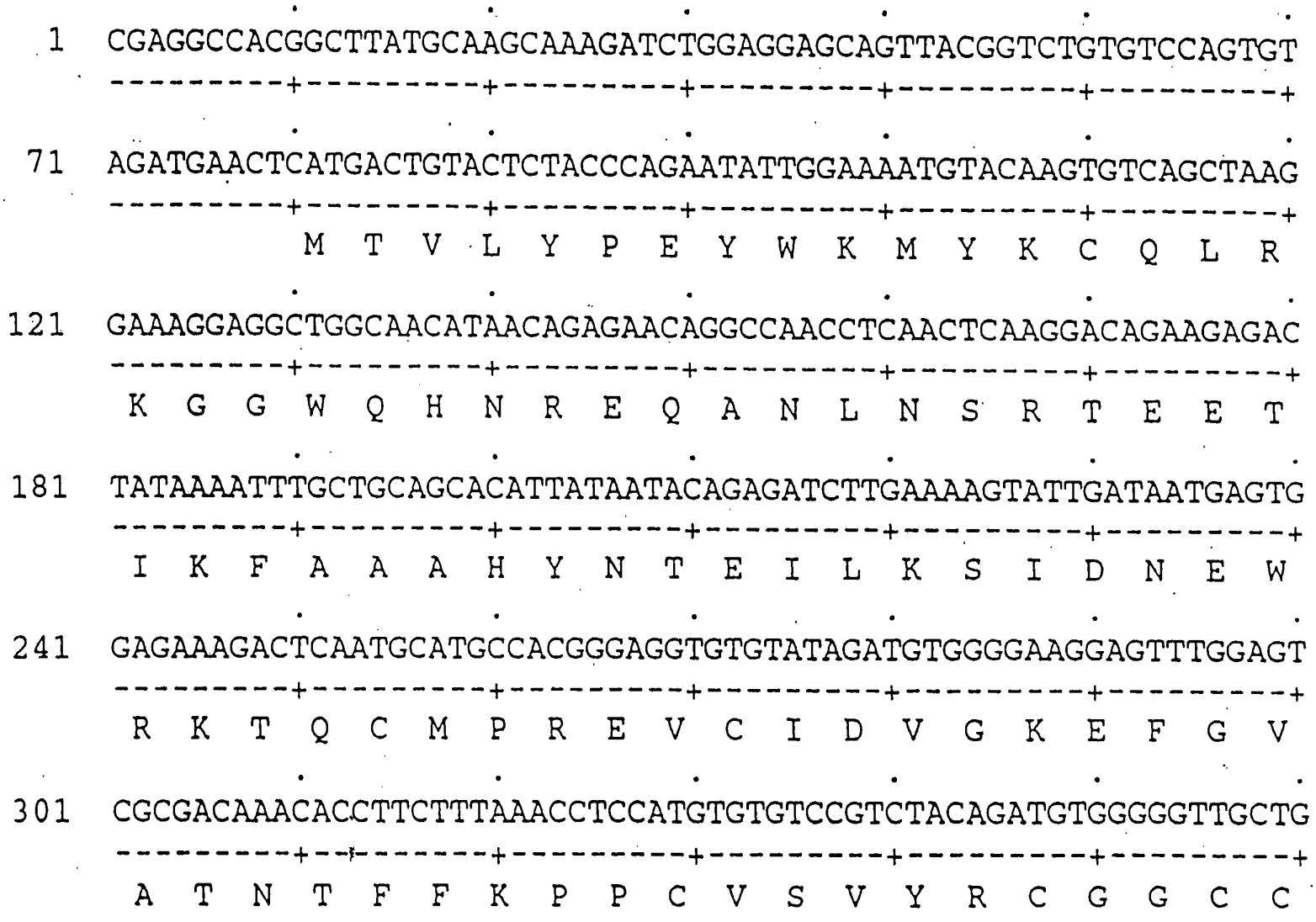

1 CGAGGCCACGGCTTATGCAAGCAAAGATCTGGAGGAGCAGTTACGGTCTGTGTCCAGTGT
M T V L Y P E Y W K M Y K C Q L R
71 AGATGAACTCATGACTGTACTCTACCCAGAATATTGGAAAATGTACAAGTGTCAGCTAAG
K G G W Q H N R E Q A N L N S R T E E T
121 GAAAGGAGGCTGGCAACATAACAGAGAACAGGCCAACCTCAACTCAAGGACAGAAGAGAC
I K F A A A H Y N T E I L K S I D N E W
181 TATAAAATTTGCTGCAGCACATTATAATACAGAGATCTTGAAAAGTATTGATAATGAGTG
R K T Q C M P R E V C I D V G K E F G V
241 GAGAAAGACTCAATGCATGCCACGGGAGGTGTGTATAGATGTGGGGAAGGAGTTTGGAGT
A T N T F F K P P C V S V Y R C G G C C
301 CGCGACAAACACCTTCTTTAAACCTCCATGTGTGTCCGTCTACAGATGTGGGGGTTGCTG

FIG. 2A

```
361  CAATAGTGAGGGGCTGCAGTGCATGAACACCAGCACGAGCTACCTCAGCAAGACGTTATT
     ---------+---------+---------+---------+---------+---------+
      N  S  E  G  L  Q  C  M  N  T  S  T  S  Y  L  S  K  T  L  F

421  TGAAATTACAGTGCCTCTCTCTCAAGGCCCCAAACCAGTAACAATCAGTTTTGCCAATCA
     ---------+---------+---------+---------+---------+---------+
      E  I  T  V  P  L  S  Q  G  P  K  P  V  T  I  S  F  A  N  H

481  CACTTCCTGCCGATGCATGTCTAAACTGGATGTTTACAGACAAGTTCATTCCATTATTAG
     ---------+---------+---------+---------+---------+---------+
      T  S  C  R  C  M  S  K  L  D  V  Y  R  Q  V  H  S  I  I  R

541  ACGTTCCCTGCCAGCAACACTACCACAGTGTCAGGCAGCGAACAAGACCTGCCCCACCAA
     ---------+---------+---------+---------+---------+---------+
      R  S  L  P  A  T  L  P  Q  C  Q  A  A  N  K  T  C  P  T  N

601  TTACATGTGGAATAATCACATCTGCAGATGCCTGGCTCAGGAAGATTTTATGTTTTCCTC
     ---------+---------+---------+---------+---------+---------+
      Y  M  W  N  N  H  I  C  R  C  L  A  Q  E  D  F  M  F  S  S

661  GGATGCTGGAGATGACTCAACAGATGGATTCCATGACATCTGTGGACCAAACAAGGAGCT
     ---------+---------+---------+---------+---------+---------+
      D  A  G  D  D  S  T  D  G  F  H  D  I  C  G  P  N  K  E  L
```

FIG.2B

```
721   GGATGAAGAGACCTGTCAGTGTGTCTGCAGAGCGGGGCTTCGGCCTGCCAGCTGTGGACC
      ---------+---------+---------+---------+---------+---------+
       D  E  E  T  C  Q  C  V  C  R  A  G  L  R  P  A  S  C  G  P

781   CCACAAAGAACTAGACAGAAACTCATGCCAGTGTGTCTGTAAAAACAAACTCTTCCCCAG
      ---------+---------+---------+---------+---------+---------+
       H  K  E  L  D  R  N  S  C  Q  C  V  C  K  N  K  L  F  P  S

841   CCAATGTGGGGCCAACCGAGAATTTGATGAAAACACATGCCAGTGTGTATGTAAAAGAAC
      ---------+---------+---------+---------+---------+---------+
       Q  C  G  A  N  R  E  F  D  E  N  T  C  Q  C  V  C  K  R  T

901   CTGCCCCAGAAATCAACCCCTAAATCCTGGAAAATGTGCCTGTGAATGTACAGAAAGTCC
      ---------+---------+---------+---------+---------+---------+
       C  P  R  N  Q  P  L  N  P  G  K  C  A  C  E  C  T  E  S  P

961   ACAGAAATGCTTGTTAAAAGGAAAGAAGTTCCACCACCAAACATGCAGCTGTTACAGACG
      ---------+---------+---------+---------+---------+---------+
       Q  K  C  L  L  K  G  K  K  F  H  H  Q  T  C  S  C  Y  R  R

1021  GCCATGTACGAACCGCCAGAAGGCTTGTGAGCCAGGATTTTCATATAGTGAAGAAGTGTG
      ---------+---------+---------+---------+---------+---------+
       P  C  T  N  R  Q  K  A  C  E  P  G  F  S  Y  S  E  E  V  C
```

FIG. 2C

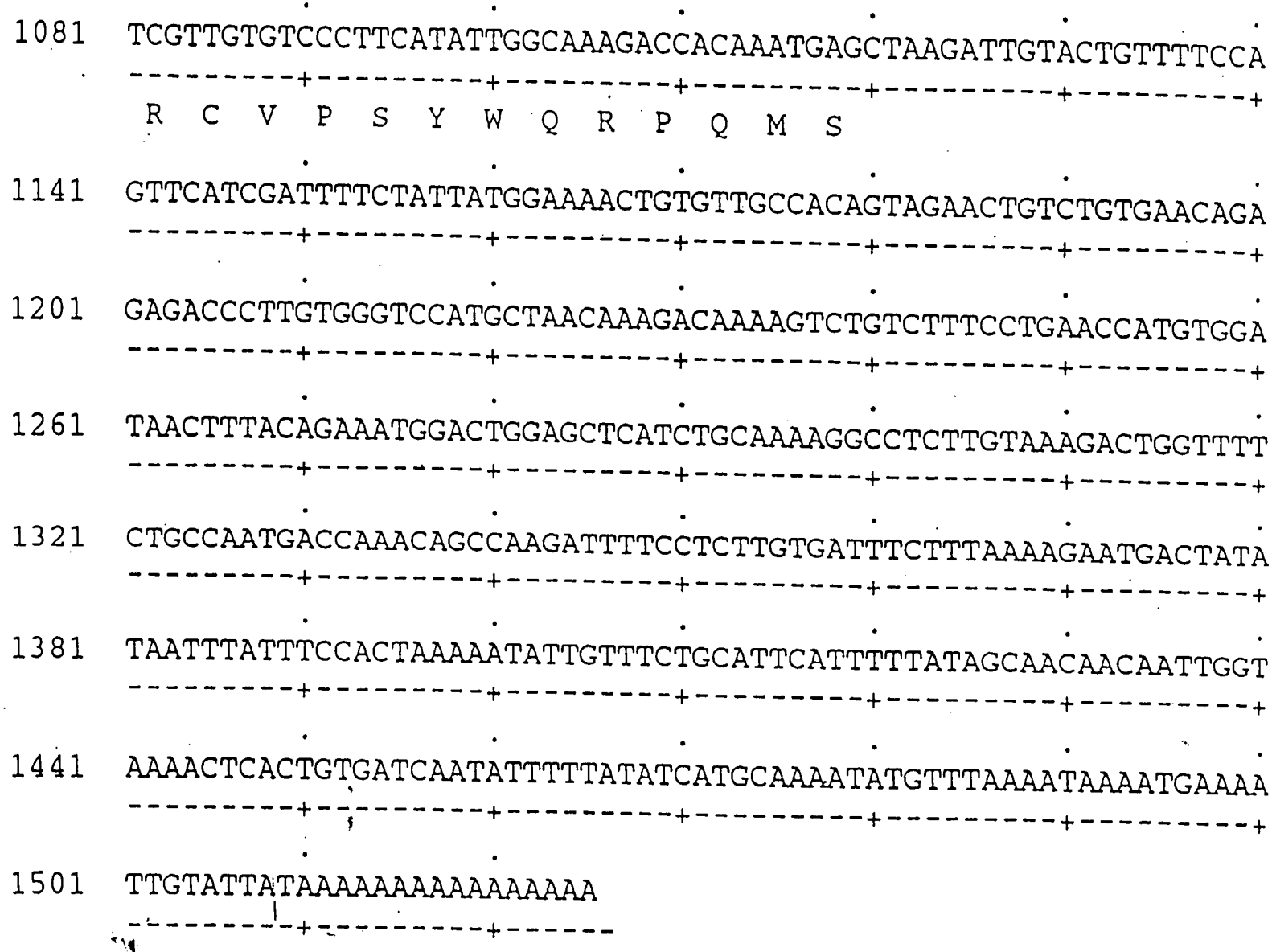

1081 TCGTTGTGTCCCTTCATATTGGCAAAGACCACAAATGAGCTAAGATTGTACTGTTTTCCA
R C V P S Y W Q R P Q M S
1141 GTTCATCGATTTTCTATTATGGAAAACTGTGTTGCCACAGTAGAACTGTCTGTGAACAGA
1201 GAGACCCTTGTGGGTCCATGCTAACAAAGACAAAAGTCTGTCTTTCCTGAACCATGTGGA
1261 TAACTTTACAGAAATGGACTGGAGCTCATCTGCAAAAGGCCTCTTGTAAAGACTGGTTTT
1321 CTGCCAATGACCAAACAGCCAAGATTTTCCTCTTGTGATTTCTTTAAAAGAATGACTATA
1381 TAATTTATTTCCACTAAAAATATTGTTTCTGCATTCATTTTTATAGCAACAACAATTGGT
1441 AAAACTCACTGTGATCAATATTTTTATATCATGCAAAATATGTTTAAAATAAAATGAAAA
1501 TTGTATTATAAAAAAAAAAAAAAA

FIG. 2D

Alignment of VEGF-2 Sequences

MHSLGFFSVACSLLAAALLPGPREAPAAAAAFESGLDLSDAEPDAGEATAYASKDLEEQLRSVSSVDELMTVLYPEYWKM SEQ ID NO:2

MTVLYPEYWKM SEQ ID NO:4

YKCQLRKGGWQHNREQANLNSRTEETIKFAAAHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVY SEQ ID NO:2

YKCQLRKGGWQHNREQANLNSRTEETIKFAAAHYNTEILKSIDNEWRKTQCMPREVCIDVGKEFGVATNTFFKPPCVSVY SEQ ID NO:4

RCGGCCNSEGLQCMNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRRSLPATLPQCQAAN SEQ ID NO:2

RCGGCCNSEGLQCMNTSTSYLSKTLFEITVPLSQGPKPVTISFANHTSCRCMSKLDVYRQVHSIIRRSLPATLPQCQAAN SEQ ID NO:4

KTCPTNYMWNNHICRCLAQEDFMFSSDAGDDSTDGFHDICGPNKELDEETCQCVCRAGLRPASCGPHKELDRNSCQCVCK SEQ ID NO:2

KTCPTNYMWNNHICRCLAQEDFMFSSDAGDDSTDGFHDICGPNKELDEETCQCVCRAGLRPASCGPHKELDRNSCQCVCK SEQ ID NO:4

NKLFPSQCGANREFDENTCQCVCKRTCPRNQPLNPGKCACECTESPQKCLLKGKKFHHQTCSCYRRPCTNRQKACEPGFS SEQ ID NO:2

NKLFPSQCGANREFDENTCQCVCKRTCPRNQPLNPGKCACECTESPQKCLLKGKKFHHQTCSCYRRPCTNRQKACEPGFS SEQ ID NO:4

YSEEVCRCVPSYWQRPQMS SEQ ID NO:2

YSEEVCRCVPSYWQRPQMS SEQ ID NO:4

Express Mail No.:_____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Hu *et al.*

Application No.: 08/107,997 Group Art Unit: 1647

Filed: June 30, 1998 Examiner: C. Saoud

For: Vascular Endothelial Growth Factor 2 Attorney Docket No.: PF112P4

DECLARATION OF STUART AARONSON UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, STUART AARONSON, do declare that:

1. I am currently the Director of the Derald H. Ruttenberg Cancer Center for the Mount Sinai Medical Center in New York, New York. Since the 1970s, my research has focused on growth factors and their role in tumorigenesis and cancer as evidenced by my curriculum vitae, attached hereto as Exhibit A, which lists the publications that I have authored or co-authored. My research in the area of the molecular biology of growth factors and their receptors, including keratinocyte growth factor, fibroblast growth factor and vascular endothelial growth factor, has encompassed mammalian models of tumorigenesis, including human tumor model systems.

2. I have been asked by the Patent Attorneys representing Human Genome Sciences ("HGS") to review the specification to U.S. application serial no. 08/207,550, filed March 8, 1994, ("the HGS patent specification") and the specification to application serial no. 08/107,997, filed June 30, 1998. I have

also been asked to review and comment on the experimental evidence provided in Dr. Susan Power's Statutory Declaration, attached hereto as Exhibit B, which has been submitted in connection with proceedings related to Australian Patent Application Au-B-696764 (73941/94) in the name of HGS, entitled "Vascular Endothelial Growth Factor-2." I have also been asked to provide my comments and opinions as to what the patent specification would provide to one familiar with the molecular biology of growth factors, *e.g.*, a post doctorate or Ph.D. candidate in a research laboratory, as of the earliest filing date of the HGS patent specification, March 1994. For purposes of this analysis, I considered not only what I knew and appreciated at the relevant time, but what was expected to be known by graduate students and postdoctoral fellows who were in my laboratory at the relevant time.

3. I have reviewed and analyzed the polynucleotide and amino acid sequence identified by HGS to encode the human VEGF-2 protein, as set forth in Figure 1 of the HGS patent specification. The HGS patent specification describes, but is not limited to, the characterization of the VEGF-2 sequence and encoded protein. The HGS patent specification describes the human VEGF-2 protein as structurally related to the PDGF/VEGF family, a known family of secreted growth factors. The HGS patent specification further discloses that the VEGF-2 polynucleotide is predicted to contain an open reading frame of approximately 1050 residues, which encodes VEGF-2. (*See* the HGS patent specification at page 5, lines 25-27). The specification reports that at the amino acid level, VEGF-2 exhibits the highest homology to vascular endothelial growth factor (30% identity), followed by PDGF alpha (23%) and PDGF beta (22%). (*See* the HGS patent specification at page 5, lines 28-31). The HGS patent specification further characterizes the VEGF-2 protein as containing eight cysteines which are conserved among all known members of the PDGF/VEGF family, and in addition, also contains the fourteen amino acid signature motif, PXCVXXXRCXGCCN, found in all members of the

PDGF/VEGF family. (*See* the HGS patent specification at page 5, lines 31-33).

4. Based on the characterization of the VEGF-2 protein set forth in the HGS patent specification, one would recognize that the protein was a member of the PDGF/VEGF family of growth factors. The PDGF/VEGF family of growth factors, like other growth factors, are normally secreted in order to exert their growth promoting or mitogenic effects. Since all previously identified members of the PDGF/VEGF family were known to be secreted, one would expect the newly identified VEGF-2 to also be secreted.

5. By March 1994 it was well known to me and, I believe to my colleagues in the angiogenic field that the PDGF/VEGF family of growth factors were expressed initially as precursor proteins which underwent proteolytic processing resulting in a mature, secreted form of the protein. Thus, I would have predicted that VEGF-2 would be processed in a similar way. The 350 amino acid sequence set forth in Figure 1 of the HGS patent specification contains the conserved, signature motifs for an active form of a protein belonging to the PDGF/VEGF family. Thus, in March 1994, I would have predicted that the protein encoded by the sequence disclosed in Figure 1, expressed and secreted by a host cell as taught in the HGS patent specification, would contain those signature motifs characteristic of the PDGF/VEGF family and be biologically active.

The HGS Patent Specification Provides Sufficient Information Such that a Molecular Biologist Recognizes the Possession of a Mature Biologically Active Form of VEGF-2

6. The question of whether the 350 amino acid sequence as set forth in the HGS patent specification does indeed contain sufficient information to result in the mature processed form of VEGF-2 when secreted from a host cell has, in my opinion, been addressed and affirmatively confirmed in the experiments

reported in Dr. Susan Power's Statutory Declaration (attached hereto as Exhibit B).

7. The experiment set forth in Dr. Power's Declaration describes the use of two constructs: the 350 amino acid sequence, containing the nucleotide sequence encoding the 350 amino acid sequence of VEGF-2 (as set forth in Figure 1 of the patent specification) in frame with a heterologous promoter and signal sequence; the 419 amino acid sequence containing the nucleotide sequence encoding the 419 amino acid sequence of VEGF-2 in frame with a heterologous promoter. These two constructs were used to transform a mammalian cell line. The cells were cultured under conditions to allow the cells to express the gene products encoded by the vectors. At various time points the cell lysates and culture medium were collected and each was assayed for the presence of VEGF-2. The presence of VEGF-2 was determined by a Western blot analysis using a polyclonal antibody to VEGF-2 that recognizes both the unprocessed precursor form as well as the processed, secreted form of VEGF-2 (*See* ¶ 13 of Dr. Power's Declaration).

8. The results of the experiments reported in Dr. Power's Declaration demonstrate that the construct expressing the 350 amino acid sequence of VEGF-2 contains sufficient information to allow for the natural and correct processing of the protein to a mature biologically active protein. Indeed, the secreted proteins which result from the expression of the constructs encoding the 419 amino acid sequence and the 350 amino acid sequence of VEGF-2 are indistinguishable in size. Both are secreted and processed to a mature form of the protein which resolves as a band at approximately 30 kDa, with another minor band detectable at approximately 21 kDa (*See* Figure 1 of Dr. Power's Declaration, at Gel 3, lanes 22 and 24). Since the filing of the HGS patent specification, the 30 kDa and the 21 kDa species have been consistently identified in the art as processed mature forms of VEGF-2 with biological activity. (*See*, Joukov *et al.*, 1997, EMBO J 16: 3898, at 3898, "Joukov").

9. In sum, the experimental evidence provided in Dr. Power's Declaration demonstrates that the constructs encoding the 350 amino acid sequence and the 419 amino acid sequence of VEGF-2 are both naturally, correctly and indistinguishably processed by the cell to mature forms of the protein.

The VEGF-2 Polypeptide Is Processed To Its Mature Form Similarly From Cell Type to Cell Type

10. Since the March 1994 filing date of the HGS patent specification, subsequent publications by the inventors and others have further characterized the proteolytic processing of VEGF-2. The inventors' own publications confirm that when expressed in mammalian cells, the precursor form of VEGF-2 undergoes proteolytic processing. (*See*, Hu et al., 1997, FASEB J. 11(6) :498-504, "Hu").

11. The capacity to be processed to the mature form of VEGF-2 is an inherent property of the VEGF-2 amino acid sequence. The only information necessary for processing VEGF-2 to its mature form is contained in the amino acid sequence of VEGF-2. Any host cell with proteolytic enzymes and cellular machinery for processing the VEGF-2 polypeptide, *i.e.* a mammalian cell, will naturally process VEGF-2 to its mature form. While the efficiency of the processing to the mature form of VEGF-2 can vary depending on the cell type from which VEGF-2 is expressed, the capacity to be processed to the mature form is similar from cell type to cell type. The Joukov publication compares the proteolytic processing of VEGF-2 expressed by a number of different cell lines, including COS cells, PC-3 cells, HT 1080 cells, and 293 EBNA cells. The results of this comparison demonstrate that the processing of the VEGF-2 polypeptide to its mature form is similar from cell to cell (Joukov, at page 3901, second column). Thus, while the efficiency of processing of the precursor form may vary from cell type to cell type, the capacity of the amino acid sequence of the precursor form of VEGF-2 which allows the protein to be naturally processed does not vary.

12. I note that Example 2 of the HGS specification reports the translation of a VEGF-2 polypeptide by an *in vitro* reticulocyte lysate system resulting in a protein having an estimated molecular weight of 36-38 kD (*See*, the HGS specification at page 28, lines 5-12). Given that an *in vitro* expression system was used to achieve this result, the reported molecular weight is not surprising or inconsistent as compared to the molecular weights reported for the mature form of VEGF-2 as processed by mammalian cells. *In vitro* translation systems may contain a subset of the proteolytic enzymes found in mammalian cells. Similarly, non-mammalian expression systems, such as bacteria or baculovirus host cells, also contain different proteolytic enzymes for the processing of precursor proteins to their mature form. Thus, when expression systems other than intact mammalian cells are used to express mammalian proteins, such as VEGF-2, it is not unexpected for the mature form of the protein to have a molecular weight differing from that observed in mammalian cells. In addition to differences in processing efficiencies there may also be post-translational modifications, including the attachment of different sugar residues to the protein, any of which may result in a variance in molecular weight as observed by SDS-PAGE. However, all of the information required to achieve the mature processed form of the protein lies in the amino acid sequence of the precursor protein. Thus, other than providing the amino acid sequence of the precursor protein, it is unnecessary to provide the mechanics of processing as this information is natural and intrinsic to the expression system being utilized.

The Mature Form of VEGF-2 As Expressed and Secreted is an Inherent Feature of the Sequence of the Precursor Polypeptide

13. I have also been asked to comment on the characterization of the inherent features of the mature form of VEGF-2 as provided by Dr. Alitalo in portions of the file history of U.S. Patent No. 6,221,839, issued April 24, 2001, from U.S. Application No. 08/510,133, filed August 1, 1995, ("the Alitalo application"). I have reviewed: (a) Dr. Alitalo's Conditional Petition to

Reverse or Withdraw Adverse Priority Determination Pursuant to 37 C.F.R. § 1.181, dated June 11, 1997 (the "Petition" attached hereto as Exhibit C) and (b) a Declaration by Dr. Carl-Henrik Heldin, executed June 4, 1997 (the "Heldin Declaration" attached hereto as Exhibit D), both provided in the prosecution of the Alitalo application.

14. According to Dr. Alitalo, the *molecular weight* of the processed mature form of VEGF-2 as assessed by SDS-PAGE under reducing conditions is an inherent property of the molecule (*See* Petition page 7, line 31 to page 8, lines 24-26). Dr. Heldin is also in agreement with this principal, stating, "[l]ike all organic chemical compounds, polypeptides may be characterized by any of several inherent physical properties, such as *molecular formula* and *molecular weight*. Such physical properties are inherent characteristics of organic molecules in that they are intrinsic properties of the molecules" (*See* Heldin Declaration § 10, at page 6, lines 12-13, emphasis added).

15. Dr. Alitalo also characterizes the processing of the precursor form of the VEGF-2 polypeptide to a *partial amino acid sequence of its precursor* as an inherent property of that polypeptide (*See* Petition page 8, lines 24-26). Dr. Heldin provides a similar characterization, in that the secreted mature form of VEGF-2 has a *partial amino acid sequence of its precursor form* and an apparent 23kD molecular weight, both of which are inherent features, intrinsic to the secreted form of the polypeptide (*See* Heldin Declaration § 11, at page 6, lines 28-30).

16. Dr. Alitalo characterizes the ability of the secreted form to contain those residues required for *activity* as another inherent feature of the VEGF-2 polypeptide (*See* Petition page 15, lines 7-15). Dr. Heldin further expands on Dr. Alitalo's characterization, stating that the secreted mature form of VEGF-2 comprises a portion of the amino acid sequence of the precursor form of VEGF-2 which binds to the Flt4 receptor tyrosine kinase and stimulates

phosphorylation thereof, and that these *activities* are inherent properties of the polypeptide (*See* Heldin Declaration § 11, at page 8, lines 2-6).

17. Thus, as Drs. Alitalo and Heldin have concluded, I would also conclude that the capacity of the precursor form of VEGF-2 to be processed to a mature form that retains a portion of the precursor amino acid sequence is a natural and intrinsic property of that amino acid sequence. It is the precursor form of the polypeptide that intrinsically contains the information required for the cell's machinery to process the protein to its mature form. Indeed, the capacity of the precursor form of VEGF-2 to be naturally processed to its mature form is an inherent property of that polypeptide, as confirmed by the experiments described in Dr. Powers' Declaration as discussed above in paragraphs 6-9. The molecular weight of the resulting processed form is an inherent property of that polypeptide as it is processed by the cell from which it is expressed. The resulting processed mature form possesses biological activity which is also an inherent property of that polypeptide.

Conclusion

18. In my opinion, the experimental evidence provided in Dr. Power's Declaration confirms the teachings of the HGS patent specification, demonstrating that the expression of the construct encoding the 350 amino acid sequence as set forth in the patent specification results in a secreted mature form of VEGF-2. Furthermore, these results confirm that the construct encoding the 350 amino acid sequence contains sufficient information to be naturally and correctly processed by the cell resulting in a mature processed VEGF-2 protein.

19. Following the teachings of the HGS patent specification, as demonstrated by Dr. Power's Declaration, I or a molecular biologist would recognize that the 350 amino acid sequence of VEGF-2 has the capacity to be processed to a mature form of VEGF-2 by the cell. The capacity of the precursor form of

VEGF-2 to be processed to a mature form is an intrinsic property of that polypeptide.

20. Therefore, a molecular biologist provided with the teaching of the HGS patent specification would be able to express the precursor form of VEGF-2 as its naturally processed mature form. Further, a molecular biologist provided with the teaching of the HGS patent specification would recognize that the biological activity and molecular weight of the resulting processed mature form of VEGF-2 are intrinsic and natural properties of that molecule. Thus, based on the teachings of the HGS patent specification, a molecular biologist would be able to identify and obtain a mature processed form of VEGF-2.

AND I declare further that all statements made in this Declaration of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Date: November 29, 2001

Stuart Aaronson

6/28/00

CURRICULUM VITAE

Name: Stuart A. Aaronson

Date and Place of Birth: February 28, 1942, Mt. Clemens, Michigan

Citizenship: U.S.A.

Marital Status: Married, three children

Education and Training:

1959-1962	B.S. (Chemistry; summa cum laude), University of California, Berkeley
1962-1966	M.D., University of California Medical School, San Francisco
1965-1966	Fellowship, Dept. of Biochemistry, University of Cambridge, Cambridge, United Kingdom
1966-1967	Intern, Medicine, Moffitt Hospital, San Francisco

Brief Chronology of Employment:

1967-1969	Staff Associate, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, MD
1969-1970	Senior Staff Fellow, Viral Carcinogenesis Branch
1970-1977	Head, Molecular Biology Section, Viral Carcinogenesis Branch
1977-1993	Chief, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland
1993-	Director, Ruttenberg Cancer Center, Mount Sinai Medical Center, New York, NY & Jane B. and Jack R. Aron, Professor of Neoplastic Diseases

Medical Licenses

New York
Virginia

Honors and Awards:

1962	Phi Beta Kappa
1966	Alpha Omega Alpha
1982	Rhoads Memorial Award
1982	PHS Meritorious Service Medal
1989	Paul Ehrlich Award
1989	PHS Distinguished Service Medal
1990	Milken Award
1991	Chirone Prize
1991	Harvey Lecture

1991 Wadsworth Memorial Foundation Award

Societies:

American Society for Microbiology
American Association for the Advancement of Science
Society for Experimental Biology and Medicine
American Association for Cancer Research, Inc.
American Society for Virology, Inc.

Memberships and Affiliations:

1975-1978 Member, Viral Cancer Program Coordinating Committee
1975-1976 Ad Hoc Member, Experimental Virology Study Section, NIH
1975-1978 Member, Viral Oncology Scientific Advisory Committee for FCRC
1976-1980 Member, Experimental Virology Study Section, NIH
1977- Member, Editorial Board, International Journal of Cancer
1977-1986 Associate Editor, Journal of the National Cancer Institute
1980-1985 Editorial Advisory Board, Biochimica et Biophysica Acta (BBA Reviews on Cancer)
1981- Associate Editor, Cancer Research
1983- Executive Committee, Duke Comprehensive Center, Duke University Medical Center
1984 Mott Selection Committee, General Motors Cancer Research Foundation
1984- Advisory Committee, Maimonides Conferences on Cancer Research
1984-1990 Editorial Board, Virus Research
1984-1987 Scientific Advisory Committee, American Cancer Society
1985-1987 External Scientific Review Committee, Comprehensive Center, The University of Alabama in Birmingham
1985- Editorial Advisory Board, Cancer and Metastasis Reviews
1985- Editorial Board, Cancer Reviews
1985-1989 Councillor, Society for Experimental Biology and Medicine
1985-1990 Extramural Advisory Board, Cancer Center, The University of Arizona
1986 Program Chairman, American Association of Cancer Research
1986 Co-organizer, Princess Takamatsu Symposium
1986- Guest Editor, Japanese Journal of Cancer Research (Gann)
1986- Editorial Board, Environmental and Occupational Health Sciences
1986-1987 Member, Advisory Committee, American Type Culture Collection
1987-1989 Editorial Advisory Board, Molecular Endocrinology
1987- Editorial Board, Oncogene
1988-1989 Advisory Editorial Board, ISI Atlas of Science: Biochemistry
1988- Member, Blood Services Scientific Council, American Red Cross
1989-1991 Editorial Board, Cancer Communications

1989-1992 Editorial Board, The New Biologist
1989 Visiting Professor, University of Texas, San Antonio
1990- Advisory Board, BBA Reviews on Cancer, Biochimica et Biophysica Acta
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1994- International Advisory Board, Tumori
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COMMONWEALTH OF AUSTRALIA

IN THE MATTER OF: Australian Patent Application 696764 (73941/94). In the name of: Human Genome Sciences Inc.

-and-

IN THE MATTER OF: Opposition thereto by Ludwig Institute for Cancer Research, under Section 59 of the Patents Act.

STATUTORY DECLARATION

I, Susan Power of Cell & Molecular Technologies, Inc., Phillipsburg, New Jersey, United States of America, declare as follows:

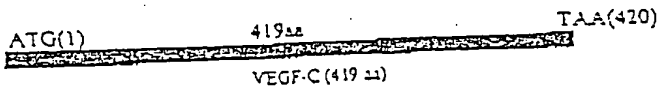
1. I am currently a Senior Molecular Biologist for Cell & Molecular Technologies, Phillipsburg, New Jersey. I have held this position since July, 1999. Prior to that appointment I was a post-doctorate research scientist, studying the molecular biology of retenoids and their role in limb bud development, at the University of Wisconsin, Madison, Wisconsin for two years. Prior to that appointment, I was a post-doctorate research scientist, studying the molecular biology of the transcription factor vHNF1 at the Pasteur Institute in Paris, France for four years. I received my Ph.D. in 1991 in Microbiology, from the National University of Ireland, Galway, Ireland.

2. The Patent Attorneys for Human Genome Sciences (HGS) requested that I perform the following experiments in order to determine whether the 350 amino acid form of VEGF-2 (corresponding to residues 70 to 419 of the 419 amino acid form of VEGF-2) fused in frame with a heterologous signal sequence would result in the expression and secretion of the protein from eukaryotic cells. They have also requested that all experiments that I conducted employ techniques routinely available by March, 1994. I have done this and the experiments I have conducted are described herein. Unless I state otherwise, all methods used herein were available prior to March, 1994.

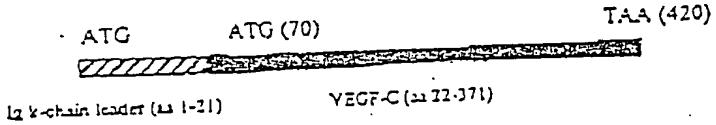
3. To determine whether the 350 amino acid form of VEGF-2 could be secreted from cells when attached to a heterologous signal sequence, I transfected eukaryotic cells with expression vectors encoding the 419 amino acid form of VEGF-2, or the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence. I grew the transfected cells under conditions to allow the cells to express the gene products encoded by the vectors. At various time points, I collected both cell lysates and culture medium and assayed for the presence of VEGF-2, in order to determine if the cells were secreting VEGF-2. The presence of VEGF-2 in either the cell lysates or culture medium was determined by Western Blot analysis of the samples using a polyclonal antibody to VEGF-2, which recognizes both the precursor form and the processed form of VEGF-2

4. The design of the expression vectors used in the study is as follows:

419 amino acid form of VEGF-2 (followed by a STOP codon at position 420):



350amino acid form of VEGF-2 linked to a heterologous signal sequence (followed by a STOP codon at position 420):



5. The nucleotide sequences encoding the 419 and the 350 amino acid forms of VEGF-2 were obtained directly from the American Type Culture Collection (ATCC). ATCC Deposit No. 97149 contains the nucleotide sequence encoding the 419 amino acid form of VEGF-2. ATCC Deposit No. 75698 contains the nucleotide sequence encoding the 350 amino acid form of VEGF-2. The nucleotide sequence encoding the 419 amino acid form of VEGF-2

was engineered to be flanked by an Eco RI site at the 5'end and a Not I site at its 3'end. The second construct contained the nucleotide sequence encoding the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, Ig k-chain leader signal sequence, a recognized signal sequence which was available as of March, 1994, and was engineered to contain a Bam HI site at its 5'end and a Not I site at its 3' end. The sequence of each of these constructs was confirmed to be correct and is detailed in Appendix I, attached hereto.

6. Each VEGF-2 construct was subcloned into the expression vector pCMV-I (see attached plasmid map, attached hereto as Appendix II), so that the VEGF-2 sequences were under the control of a CMV-I promoter, a promoter routinely used as of March, 1994. The 419 amino acid form of VEGF-2 was subcloned into the Eco RI/Not I sites of pCMV-I, while the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, was subcloned into the Bam HI/Not I sites of pCMV-I (see plasmid map, Appendix II).

7. The two VEGF-2 constructs were transiently transfected in duplicate, using the lipofectin method, comparable methods were routinely used as of March, 1994, into the Human Embryonic Kidney cell line, HEK-293 tsA-0, a cell line which was also routinely used as of March, 1994. As a control for transfection efficiency, each construct was co-transfected with the vector pCMV-β-gal. The efficiency of transfection was determined by β-gal staining 48 hours after transfection. As a negative control the vector pCMV-I without an insert was transfected in parallel.

8. The transfection design is as follows:
10 dishes transfected with: pCMV-I-VEGF-419;
10 dishes transfected with: pCMV-I-signal sequence-VEGF-350;
10 dishes transfected: pCMV-I ;
2 dishes transfected with: pCMV-I-VEGF-419 + pCMV-β-gal; and
2 dishes transfected with: pCMV-I-signal sequence-VEGF-350 + pCMV-β-gal.

9. After transfection, DMEM medium containing 3% serum was added to the cells. Aliquots of cell extracts and conditioned medium were prepared from each transfection at: T_0 hours, T_{16} hours, T_{24} hours, T_{48} hours and T_{72} hours, in duplicate.

10. At the time of harvesting the cells and medium were treated as follows:
Medium: Harvested medium was concentrated 3 fold using Centricon 10 concentrator devices. One volume of 2 x PAGE loading dye was added to each sample.
Cell Extracts: The cells were harvested by trypsinization and collected by centrifugation. The cell pellet was resuspended and lysed in 250µl of 1x PAGE loading buffer.

11. To determine the transfection efficiency, dishes transfected with the pCMV-β-gal construct were fixed and stained for β-gal activity 48 hours after transfection. All dishes were found to have the same percentage of transfected cells (70%).

12. Each protein sample was subjected to Western analysis as outlined below. Prior to loading on to a 12% (w/v) Tris-Glycine SDS-Polyacrylamide gel, the samples were boiled for 5 minutes and cooled on ice. The two end lanes of each gel contained the appropriately sized molecular weight markers to estimate the migration rate of proteins predicted to run in the 16 to 85 kDa size range. The samples were electrophoresed according to standard conditions.

13. Following electrophoresis, the samples were transferred to a PVDF membrane. Each membrane was blocked by a one hour incubation in phosphate buffered saline (PBS) containing 3% Bovine Serum Albumin (BSA). The blot was then incubated at 4°C in PBS containing 0.1% BSA and 500ng/ul of purified rabbit anti-VEGF-2 antibody, a polyclonal antibody which recognizes the precursor form and the processed form of VEGF-2. After three 5 minute washes in PBS containing 0.1% Tween, the blot was incubated for 1 hour in PBS containing 0.1% BSA and a 1:3000 dilution of Goat Anti-Rabbit IgG

Horse Radish Peroxidase (HRP) conjugated antibody. The blot was washed three times for 5 minutes in PBS containing 0.1% BSA. The blot was developed with 2ml/blot of ECL detection reagent (obtained from Amersham) for one minute and then exposed directly to Polaroid films for approximately 5 seconds.

14. The result of the experiment is shown in Figure 1, attached hereto. The samples included in the figure are as follows:

Immunoblot analysis of VEGF derivatives transiently expressed in HEK293T cells

Lane	Pellet/Supernatant	350aa -signal / 419aa	T (h) post-transfection
Gel 1			
1	P	419	24
2	S	419	24
3	P	350-signal	24
4	S	350-signal	24
5	P	350-signal	24
6	S	350-signal	24
7	P	negative control	24
8	S	positive control	48
Gel 2			
9	P	419	48
10	S	419	48
11	P	419	48
12	S	419	48
13	P	350-signal	48
14	S	350-signal	48

15	P	350-signal	48
16	S	350-signal	48
17	P	negative control	
18	S	negative control	
Gel 3			
19	P	419	72
20	S	419	72
21	P	419	72
22	S	419	72
23	P	350-signal	72
24	S	350-signal	72
25	S	350-signal	72
26	P	350-signal	72

15. The Western Blot analysis indicates that a doublet of approximately 30kDa was present in the medium collected from the transfection of both the 419 amino acid form of VEGF-2 and the 350 amino acid-VEGF-2 signal sequence constructs (see Figure 1). The secreted protein was visible beginning at 16 hours after transfection. The secreted product from cells containing the 419 amino acid construct and the 350 amino acid-VEGF2 signal sequence construct are approximately the same size.

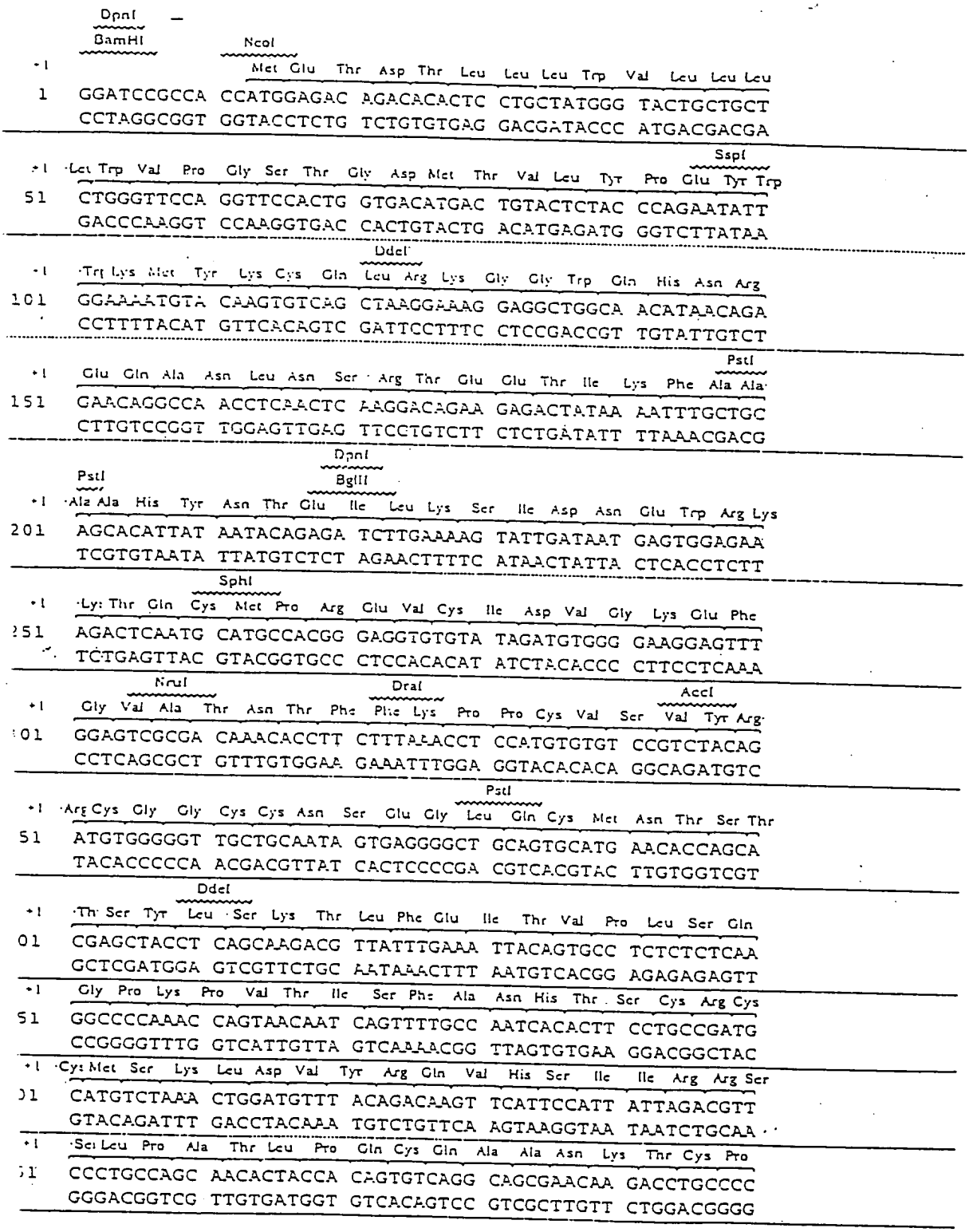
AND I declare that all the statements made in this Declaration are of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Sworn by the said Susan Power, Susan Power at Phillipsburg, New Jersey, on this 5th day of December 2000; before me Maryann White.

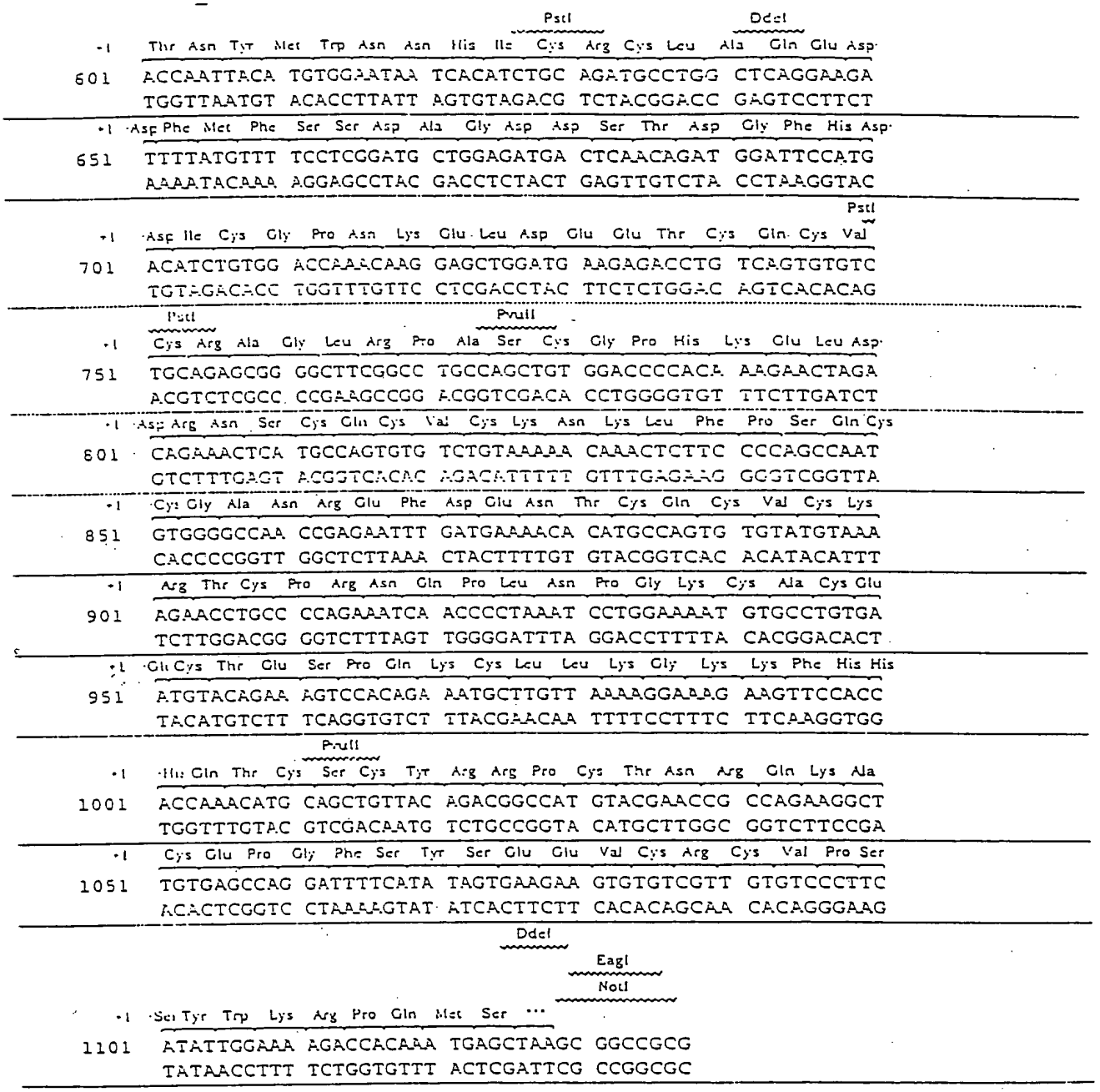
Notary Public

MARYANN WHITE
NOTARY PUBLIC, State of New York
No. 4883761
Qualified in Nassau County
Certification Filed in New York County
Commission Expires January 26, 2001

VEGF-2 350aa +Signal Sequence:



Appendix I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DdeI
+1 Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala Gln Glu Asp
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TGGTTAATGT ACACCTTATT AGTGTAGACG TCTACGGACC GAGTCCTTCT
+1 Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His Asp
651 TTTTATGTTT TCCTCGGATG CTGGAGATGA CTCAACAGAT GGATTCCATG
AAAATACAAA AGGAGCCTAC GACCTCTACT GAGTTGTCTA CCTAAGGTAC
PstI
+1 Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val
701 ACATCTGTGG ACCAAACAAG GAGCTGGATG AAGAGACCTG TCAGTGTGTC
TGTAGACACC TGGTTTGTTC CTCGACCTAC TTCTCTGGAC AGTCACACAG
PstI
PvuII
+1 Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu Leu Asp
751 TGCAGAGCGG GGCTTCGGCC TGCCAGCTGT GGACCCCACA AAGAACTAGA
ACGTCTCGCC CCGAAGCCGG ACGGTCGACA CCTGGGGTGT TTCTTGATCT
+1 Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys
801 CAGAAACTCA TGCCAGTGTG TCTGTAAAAA CAAACTCTTC CCCAGCCAAT
GTCTTTGAGT ACGGTCACAC AGACATTTTT GTTTGAGAAG GGGTCGGTTA
+1 Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys Lys
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CACCCCGGTT GGCTCTTAAA CTACTTTTGT GTACGGTCAC ACATACATTT
+1 Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys Glu
901 AGAACCTGCC CCAGAAATCA ACCCCTAAAT CCTGGAAAAT GTGCCTGTGA
TCTTGGACGG GGTCTTTAGT TGGGGATTTA GGACCTTTTA CACGGACACT
+1 Gln Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly Lys Lys Phe His His
951 ATGTACAGAA AGTCCACAGA AATGCTTGTT AAAAGGAAAG AAGTTCCACC
TACATGTCTT TCAGGTGTCT TTACGAACAA TTTTCCTTTC TTCAAGGTGG
PvuII
+1 His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala
1001 ACCAAACATG CAGCTGTTAC AGACGGCCAT GTACGAACCG CCAGAAGGCT
TGGTTTGTAC GTCGACAATG TCTGCCGGTA CATGCTTGGC GGTCTTCCGA
+1 Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser
1051 TGTGAGCCAG GATTTTCATA TAGTGAAGAA GTGTGTCGTT GTGTCCCTTC
ACACTCGGTC CTAAAAGTAT ATCACTTCTT CACACAGCAA CACAGGGAAG
DdeI
EagI
NotI
+1 Ser Tyr Trp Lys Arg Pro Gln Met Ser ***
1101 ATATTGGAAA AGACCACAAA TGAGCTAAGC GGCCGCG
TATAACCTTT TCTGGTGTTT ACTCGATTCG CCGGCGC

VEGF-2 419aa Sequence:

```
                EcoRI
+1
1   GAATTCGTGG GTCCTTCCAC CATGCACTTG CTGGGCTTCT TCTCTGTGGC
    CTTAAGCACC CAGGAAGGTG GTACGTGAAC GACCCGAAGA AGAGACACCG
                                Met His Leu Leu Gly Phe Phe Ser Val Ala

                                        SmaI
                                        XmaI
                                        AvaI          NruI
+1  Ala Cys Ser Leu Leu Ala Ala Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala
51  GTGTTCTCTG CTCGCCGCTG CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG
    CACAAGAGAC GAGCGGCGAC GCGACGAGGG CCCAGGAGCG CTCCGCGGGC
+1  Ala Ala Ala Ala Ala Phe Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro
101 CCGCCGCCGC CGCCTTCGAG TCCGGACTCG ACCTCTCGGA CGCGGAGCCC
    GGCGGCGGCG GCGGAAGCTC AGGCCTGAGC TGGAGAGCCT GCGCCTCGGG
                                        DpnI
                                        BglII
+1  Asp Ala Gly Glu Ala Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu
151 GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG AGGAGCAGTT
    CTGCGCCCGC TCCGGTGCCG AATACGTTCG TTTCTAGACC TCCTCGTCAA
                                                        SspI
+1  Leu Arg Ser Val Ser Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr
201 ACGGTCTGTG TCCAGTGTAG ATGAACTCAT GACTGTACTC TACCCAGAAT
    TGCCAGACAC AGGTCACATC TACTTGAGTA CTGACATGAG ATGGGTCTTA
            SspI                DdeI
+1  Tyr Trp Lys Met Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn
251 ATTGGAAAAT GTACAAGTGT CAGCTAAGGA AAGGAGGCTG GCAACATAAC
    TAACCTTTTA CATGTTCACA GTCGATTCCT TTCCTCCGAC CGTTGTATTG
                                                        PstI
+1  Arg Glu Gln Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala
301 AGAGAACAGG CCAACCTCAA CTCAAGGACA GAAGAGACTA TAAAATTTGC
    TCTCTTGTCC GGTTGGAGTT GAGTTCCTGT CTTCTCTGAT ATTTTAAACG
        PstI                DpnI
                            BglII
+1  Ala Ala Ala His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg
351 TGCAGCACAT TATAATACAG AGATCTTGAA AAGTATTGAT AATGAGTGGA
    ACGTCGTGTA ATATTATGTC TCTAGAACTT TTCATAACTA TTACTCACCT
                        SphI
+1  Arg Lys Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu
401 GAAAGACTCA ATGCATGCCA CGGGAGGTGT GTATAGATGT GGGGAAGGAG
    CTTTCTGAGT TACGTACGGT GCCCTCCACA CATATCTACA CCCCTTCCTC
                NruI                DraI                    AccI
+1  Phe Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
451 TTTGGAGTCG CGACAAACAC CTTCTTTAAA CCTCCATGTG TGTCCGTCTA
    AAACCTCAGC GCTGTTTGTG GAAGAAATTT GGAGGTACAC ACAGGCAGAT
    AccI                                PstI
+1  Tyr Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser
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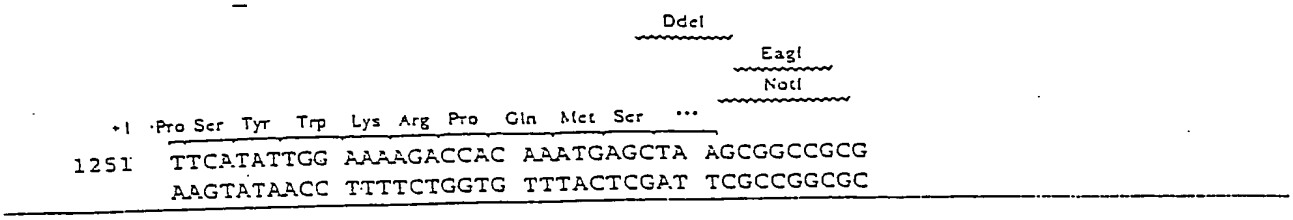
```


DdeI
EagI
NotI
+1 Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser ...
1251 TTCATATTGG AAAAGACCAC AAATGAGCTA AGCGGCCGCG
AAGTATAACC TTTTCTGGTG TTTACTCGAT TCGCCGGCGC

STRAIN SHEET

STRAIN CMV I STORAGE NUMBER 11:64

PEDIGREE:

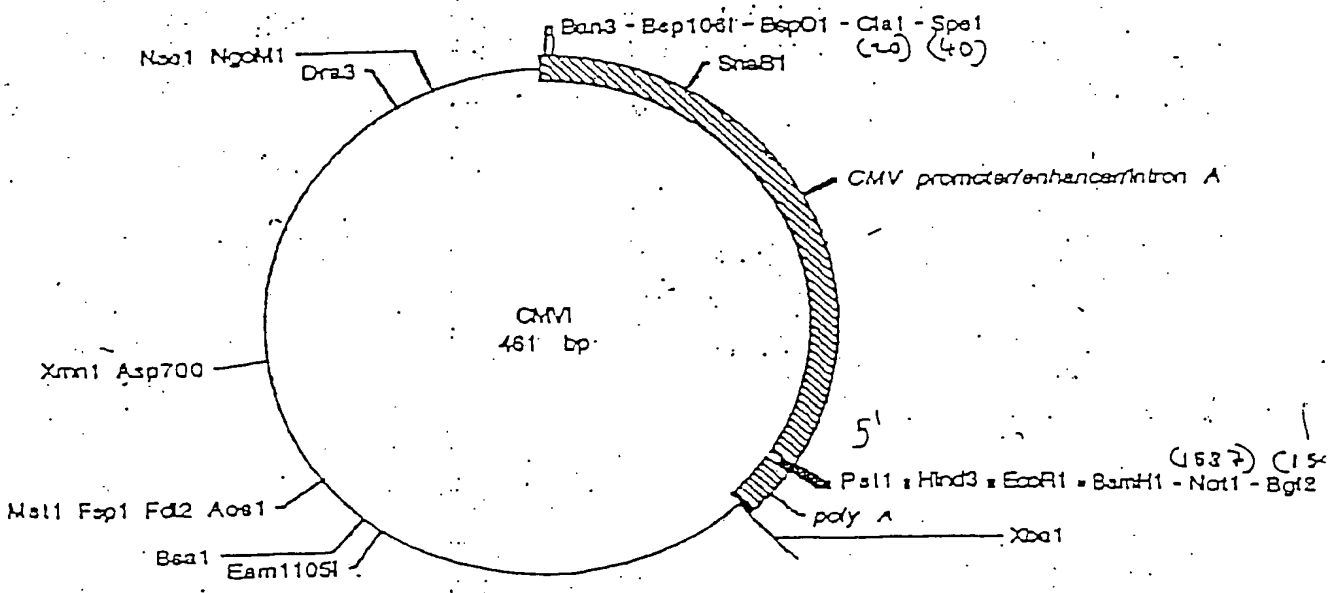
-CMVI was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMVI is 4613 bp.

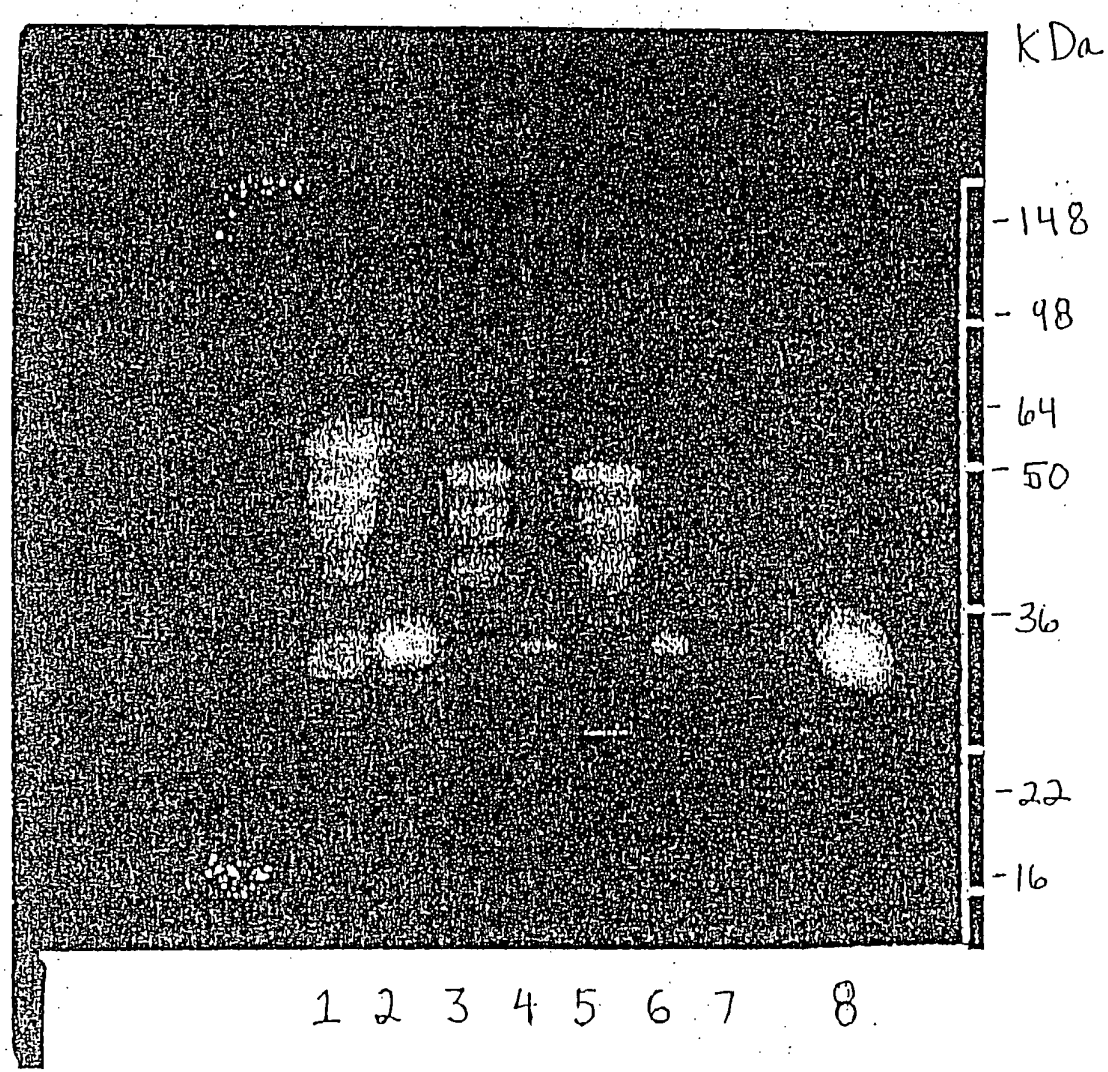
-CMVI uses ampicillin resistance.

-CMV promoter/enhancer + Intron A: nt #'s 1-1566.
-Polylinker: nt #'s 1566-1597 (5'- Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').
-SV40 polyA addition sequence: nt #'s 1598-1745.
-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

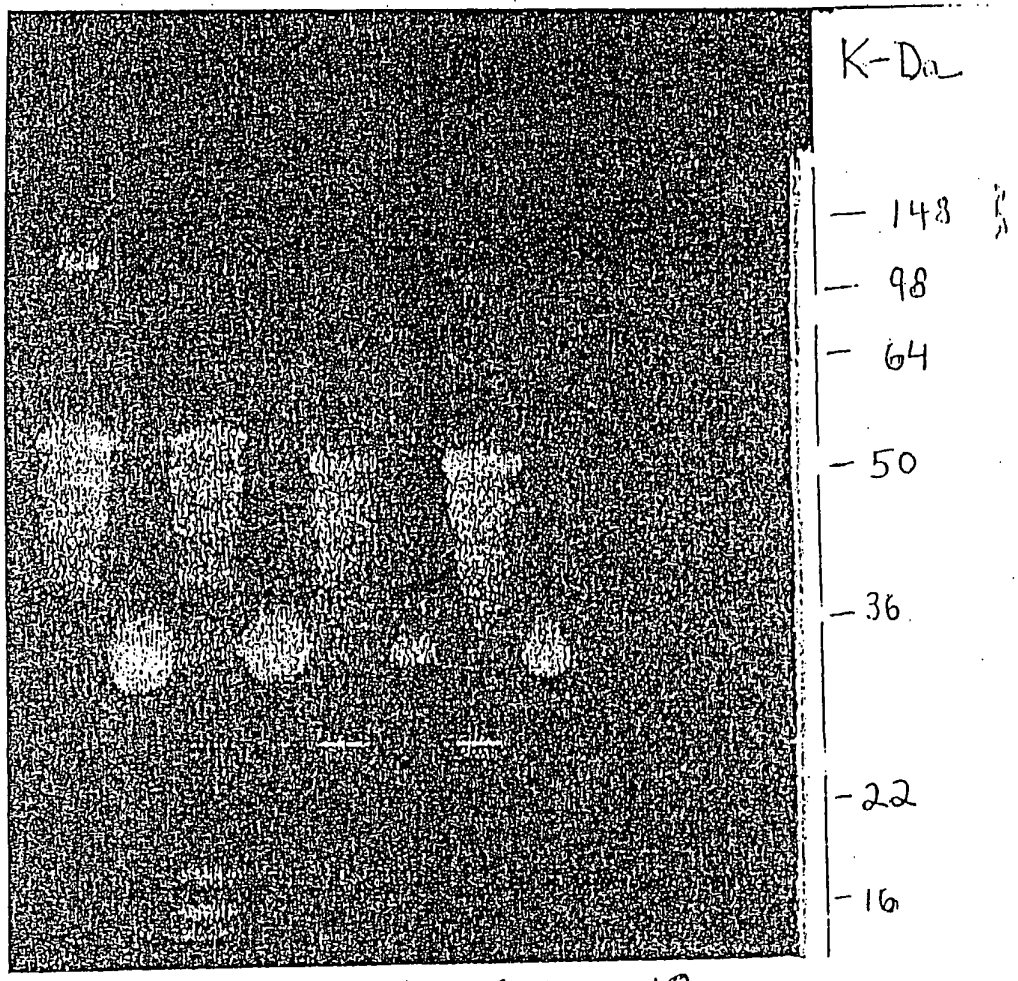
GROWTH REQUIREMENTS:

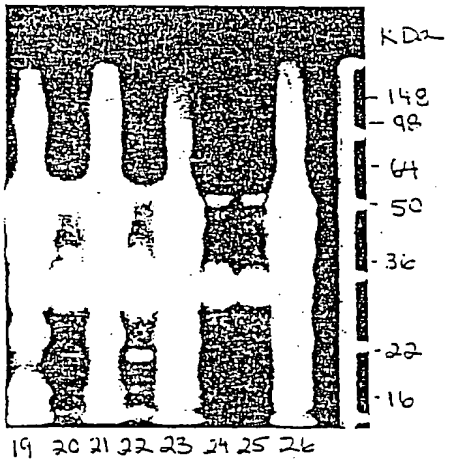


Appendix II



Power Declaration


K-Da
148
98
64
50
36
22
16
9 10 11 12 13 14 15 16 17 18

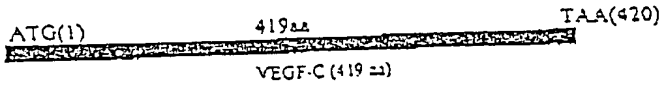


Power Declaration
Figure 1 -- Gel 3

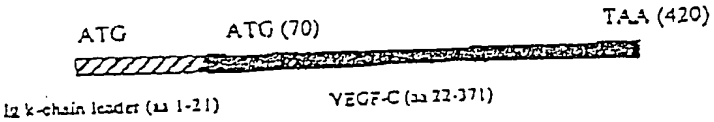
3. To determine whether the 350 amino acid form of VEGF-2 could be secreted from cells when attached to a heterologous signal sequence, I transfected eukaryotic cells with expression vectors encoding the 419 amino acid form of VEGF-2, or the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence. I grew the transfected cells under conditions to allow the cells to express the gene products encoded by the vectors. At various time points, I collected both cell lysates and culture medium and assayed for the presence of VEGF-2, in order to determine if the cells were secreting VEGF-2. The presence of VEGF-2 in either the cell lysates or culture medium was determined by Western Blot analysis of the samples using a polyclonal antibody to VEGF-2, which recognizes both the precursor form and the processed form of VEGF-2

4. The design of the expression vectors used in the study is as follows:

419 amino acid form of VEGF-2 (followed by a STOP codon at position 420):



350amino acid form of VEGF-2 linked to a heterologous signal sequence (followed by a STOP codon at position 420):



5. The nucleotide sequences encoding the 419 and the 350 amino acid forms of VEGF-2 were obtained directly from the American Type Culture Collection (ATCC). ATCC Deposit No. 97149 contains the nucleotide sequence encoding the 419 amino acid form of VEGF-2. ATCC Deposit No. 75698 contains the nucleotide sequence encoding the 350 amino acid form of VEGF-2. The nucleotide sequence encoding the 419 amino acid form of VEGF-2

was engineered to be flanked by an Eco RI site at the 5'end and a Not I site at its 3'end. The second construct contained the nucleotide sequence encoding the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, Ig k-chain leader signal sequence, a recognized signal sequence which was available as of March, 1994, and was engineered to contain a Bam HI site at its 5'end and a Not I site at its 3' end. The sequence of each of these constructs was confirmed to be correct and is detailed in Appendix I, attached hereto.

6. Each VEGF-2 construct was subcloned into the expression vector pCMV-I (see attached plasmid map, attached hereto as Appendix II), so that the VEGF-2 sequences were under the control of a CMV-I promoter, a promoter routinely used as of March, 1994. The 419 amino acid form of VEGF-2 was subcloned into the Eco RI/Not I sites of pCMV-I, while the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, was subcloned into the Bam HI/Not I sites of pCMV-I (see plasmid map, Appendix II).

7. The two VEGF-2 constructs were transiently transfected in duplicate, using the lipofectin method, comparable methods were routinely used as of March, 1994, into the Human Embryonic Kidney cell line, HEK-293 tsA-0, a cell line which was also routinely used as of March, 1994. As a control for transfection efficiency, each construct was co-transfected with the vector pCMV-β-gal. The efficiency of transfection was determined by β-gal staining 48 hours after transfection. As a negative control the vector pCMV-I without an insert was transfected in parallel.

8. The transfection design is as follows:
10 dishes transfected with: pCMV-I-VEGF-419;
10 dishes transfected with: pCMV-I-signal sequence-VEGF-350;
10 dishes transfected: pCMV-I ;
2 dishes transfected with: pCMV-I-VEGF-419 + pCMV-β-gal; and
2 dishes transfected with: pCMV-I-signal sequence-VEGF-350 + pCMV-β-gal.

9. After transfection, DMEM medium containing 3% serum was added to the cells. Aliquots of cell extracts and conditioned medium were prepared from each transfection at: T_0 hours, T_{16} hours, T_{24} hours, T_{48} hours and T_{72} hours, in duplicate.

10. At the time of harvesting the cells and medium were treated as follows:
Medium: Harvested medium was concentrated 3 fold using Centricon 10 concentrator devices. One volume of 2 x PAGE loading dye was added to each sample.
Cell Extracts: The cells were harvested by trypsinization and collected by centrifugation. The cell pellet was resuspended and lysed in 250µl of 1x PAGE loading buffer.

11. To determine the transfection efficiency, dishes transfected with the pCMV-β-gal construct were fixed and stained for β-gal activity 48 hours after transfection. All dishes were found to have the same percentage of transfected cells (70%).

12. Each protein sample was subjected to Western analysis as outlined below. Prior to loading on to a 12% (w/v) Tris-Glycine SDS-Polyacrylamide gel, the samples were boiled for 5 minutes and cooled on ice. The two end lanes of each gel contained the appropriately sized molecular weight markers to estimate the migration rate of proteins predicted to run in the 16 to 85 kDa size range. The samples were electrophoresed according to standard conditions.

13. Following electrophoresis, the samples were transferred to a PVDF membrane. Each membrane was blocked by a one hour incubation in phosphate buffered saline (PBS) containing 3% Bovine Serum Albumin (BSA). The blot was then incubated at 4°C in PBS containing 0.1% BSA and 500ng/ul of purified rabbit anti-VEGF-2 antibody, a polyclonal antibody which recognizes the precursor form and the processed form of VEGF-2. After three 5 minute washes in PBS containing 0.1% Tween, the blot was incubated for 1 hour in PBS containing 0.1% BSA and a 1:3000 dilution of Goat Anti-Rabbit IgG

Horse Radish Peroxidase (HRP) conjugated antibody. The blot was washed three times for 5 minutes in PBS containing 0.1% BSA. The blot was developed with 2ml/blot of ECL detection reagent (obtained from Amersham) for one minute and then exposed directly to Polaroid films for approximately 5 seconds.

14. The result of the experiment is shown in Figure 1, attached hereto. The samples included in the figure are as follows:

Immunoblot analysis of VEGF derivatives transiently expressed in HEK293T cells

Lane	Pellet/Supernatant	350aa -signal / 419aa	T (h) post-transfection
Gel 1			
1	P	419	24
2	S	419	24
3	P	350-signal	24
4	S	350-signal	24
5	P	350-signal	24
6	S	350-signal	24
7	P	negative control	24
8	S	positive control	48
Gel 2			
9	P	419	48
10	S	419	48
11	P	419	48
12	S	419	48
13	P	350-signal	48
14	S	350-signal	48

15	P	350-signal	48
16	S	350-signal	48
17	P	negative control	
18	S	negative control	
Gel 3			
19	P	419	72
20	S	419	72
21	P	419	72
22	S	419	72
23	P	350-signal	72
24	S	350-signal	72
25	S	350-signal	72
26	P	350-signal	72

15. The Western Blot analysis indicates that a doublet of approximately 30kDa was present in the medium collected from the transfection of both the 419 amino acid form of VEGF-2 and the 350 amino acid-VEGF-2 signal sequence constructs (see Figure 1). The secreted protein was visible beginning at 16 hours after transfection. The secreted product from cells containing the 419 amino acid construct and the 350 amino acid-VEGF2 signal sequence construct are approximately the same size.

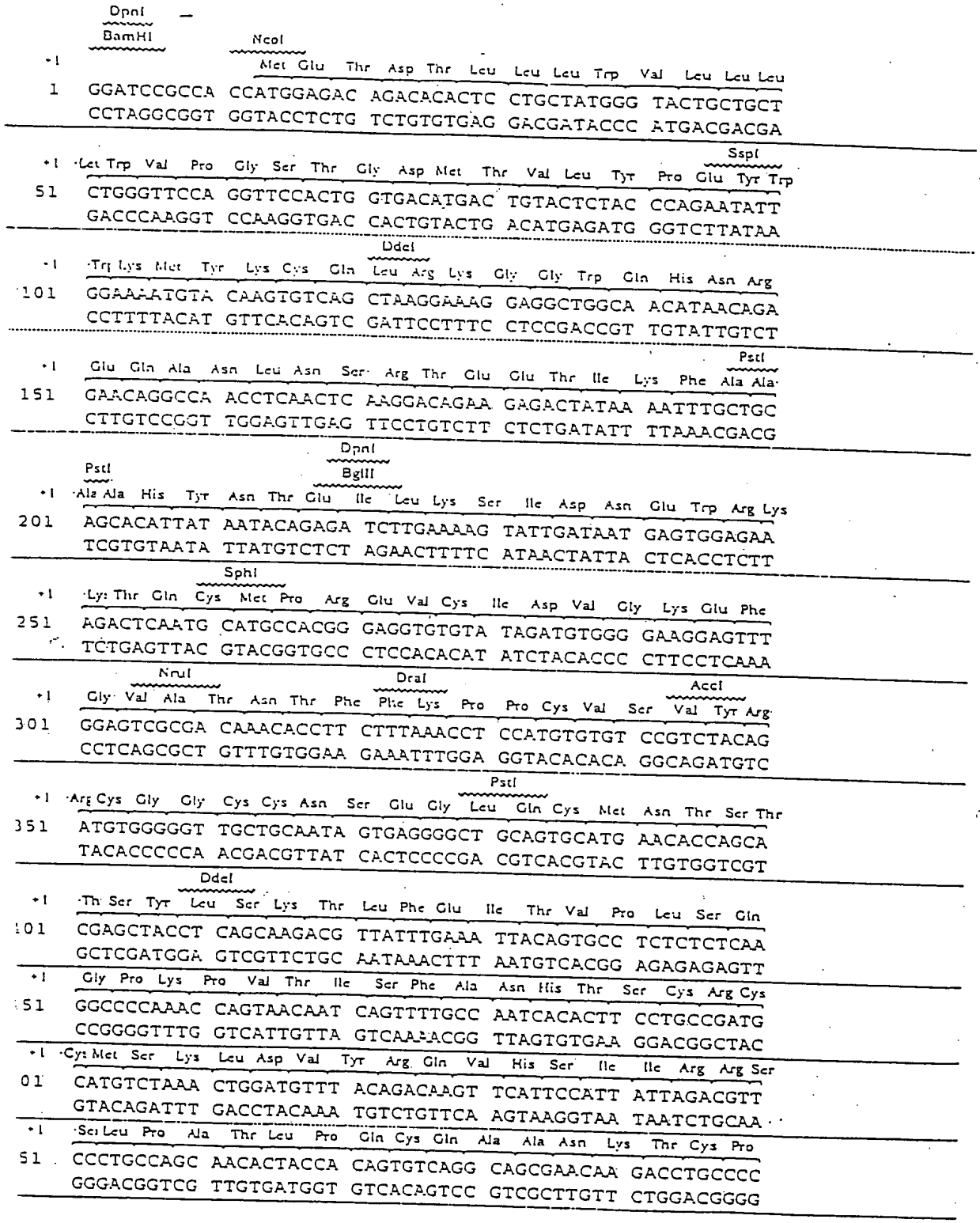
AND I declare that all the statements made in this Declaration are of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Sworn by the said Susan Power, Susan Power at Phillipsburg, New Jersey, on this 5th day of December 2000; before me Maryann White.

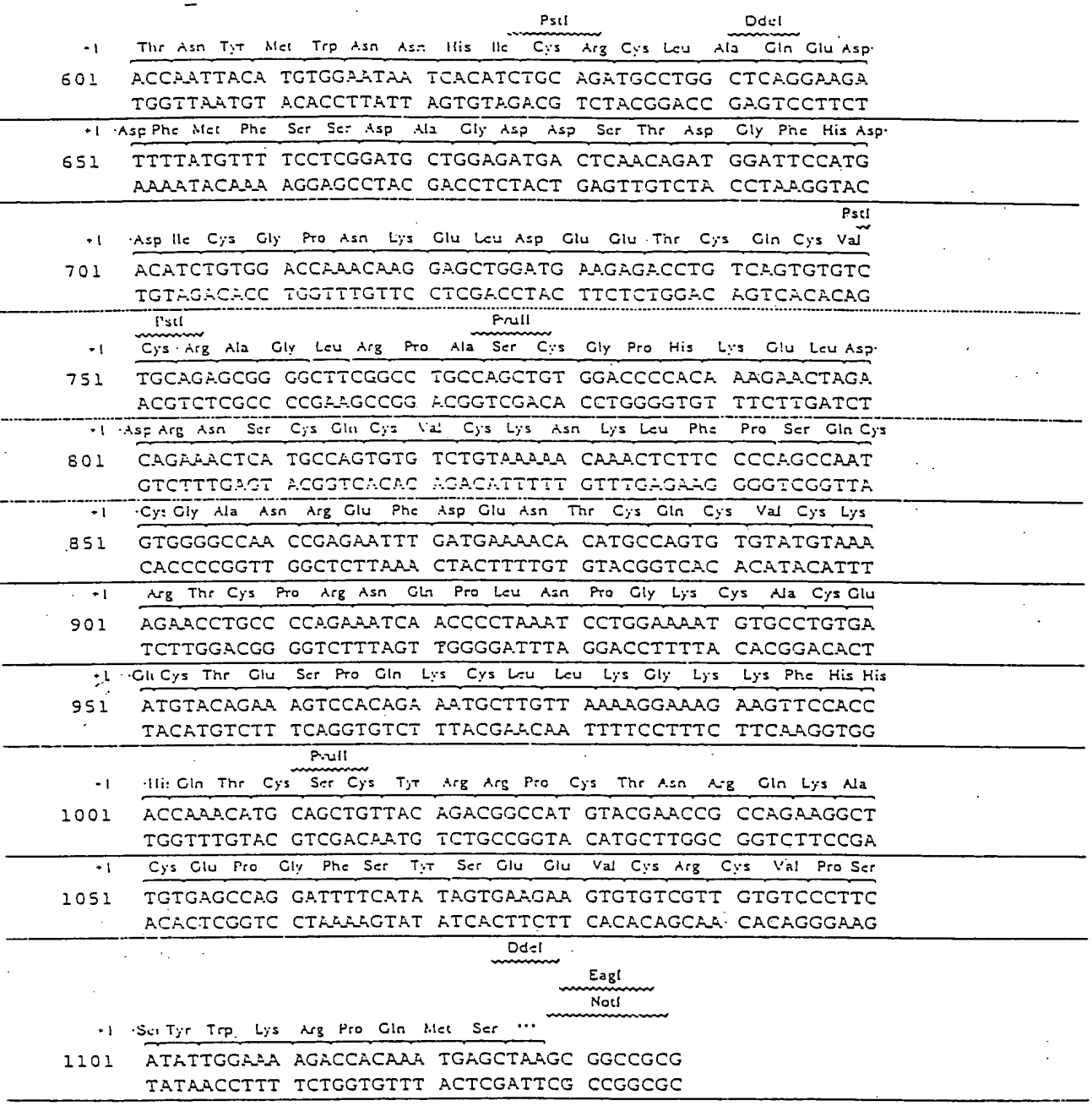
Notary Public

MARYANN WHITE
NOTARY PUBLIC, State of New York
No. 4883761
Qualified in Nassau County
Certification Filed in New York County
Commission Expires January 26, 2001

VEGF-2 350aa +Signal Sequence:



Appendix I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TGTAGACACC TGGTTTGTTC CTCGACCTAC TTCTCTGGAC AGTCACACAG
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+1 Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys
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EagI
NotI
+1 Ser Tyr Trp Lys Arg Pro Gln Met Ser ***
1101 ATATTGGAAA AGACCACAAA TGAGCTAAGC GGCCGCG
TATAACCTTT TCTGGTGTTT ACTCGATTCG CCGGCGC

VEGF-2 419aa Sequence:

```
     EcoRI
+1                                 Met His Leu Leu Gly Phe Phe Ser Val Ala
1    GAATTCGTGG GTCCTTCCAC CATGCACTTG CTGGGCTTCT TCTCTGTGGC
     CTTAAGCACC CAGGAAGGTG GTACGTGAAC GACCCGAAGA AGAGACACCG

                                      SmaI
                                      XmaI
                                      AvaI      NruI
+1   Ala Cys Ser Leu Leu Ala Ala Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala
51   GTGTTCTCTG CTCGCCGCTG CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG
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+1   Ala Ala Ala Ala Ala Phe Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro
101  CCGCCGCCGC CGCCTTCGAG TCCGGACTCG ACCTCTCGGA CGCGGAGCCC
     GGCGGCGGCG GCGGAAGCTC AGGCCTGAGC TGGAGAGCCT GCGCCTCGGG

                                          DpnI
                                          BglII
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151  GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG AGGAGCAGTT
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                                                            SspI
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     SspI                     DdeI
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                                                            PstI
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     AccI                                PstI
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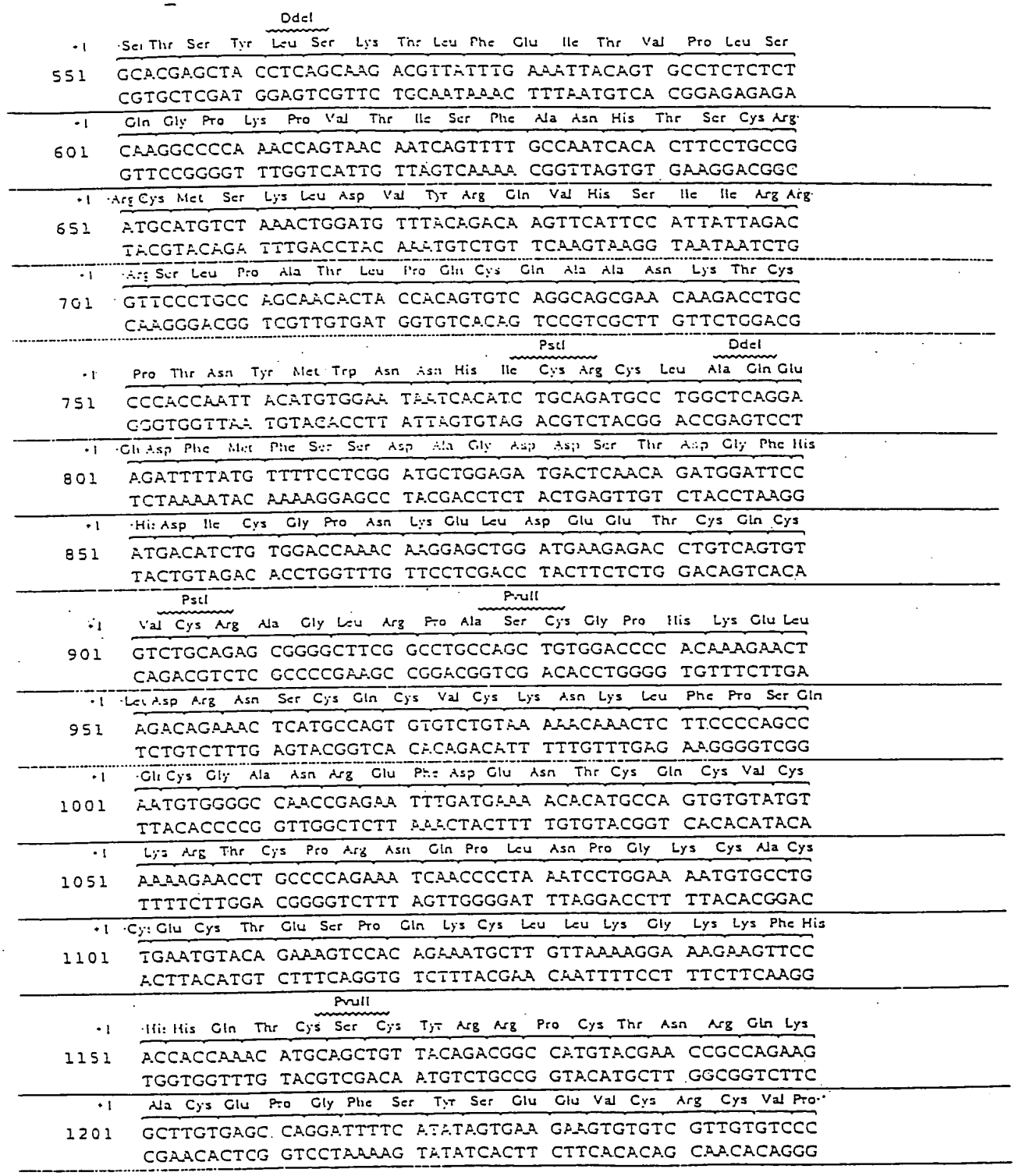
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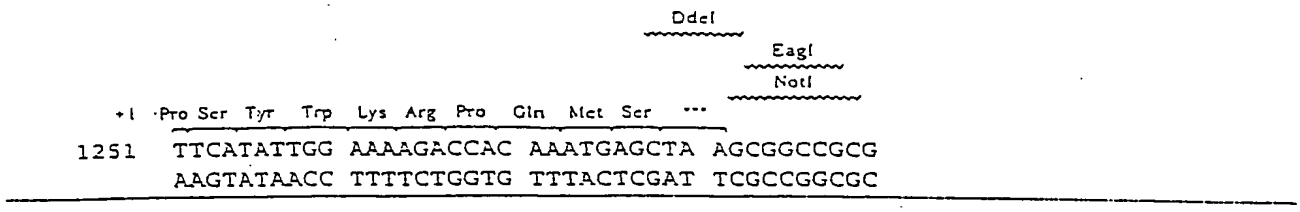
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STRAIN SHEET

STRAIN CMV I STORAGE NUMBER 11:64

PEDIGREE:

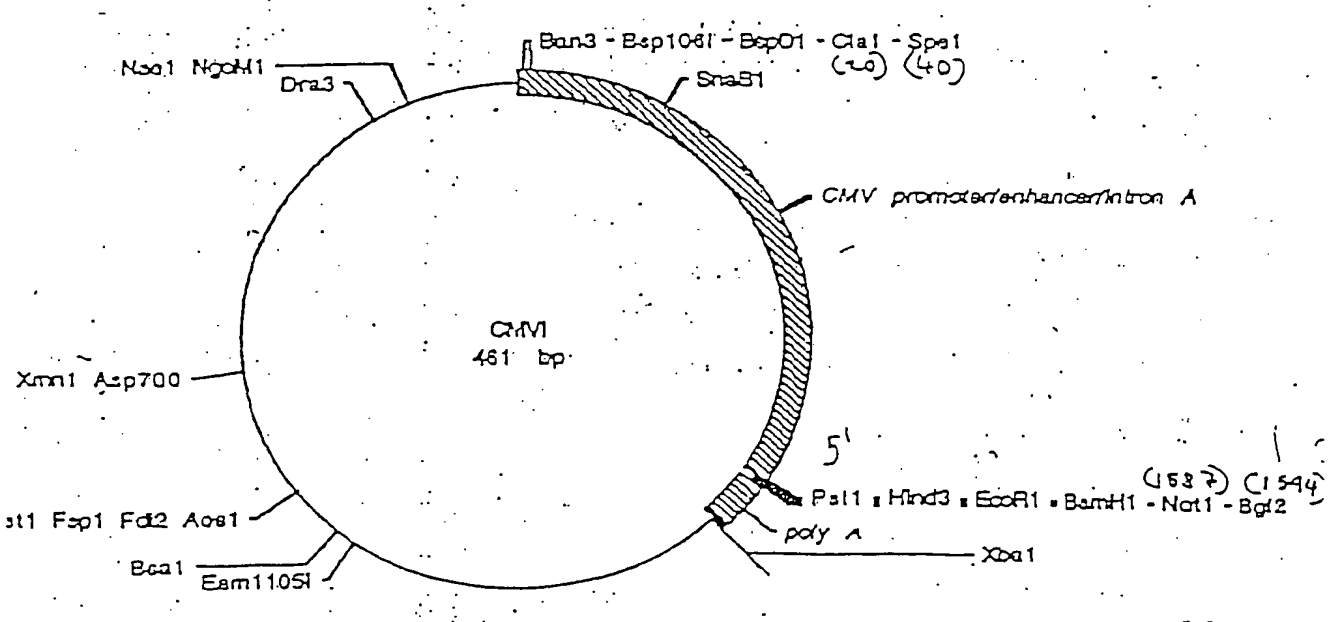
-CMVI was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMVI is 4613 bp.

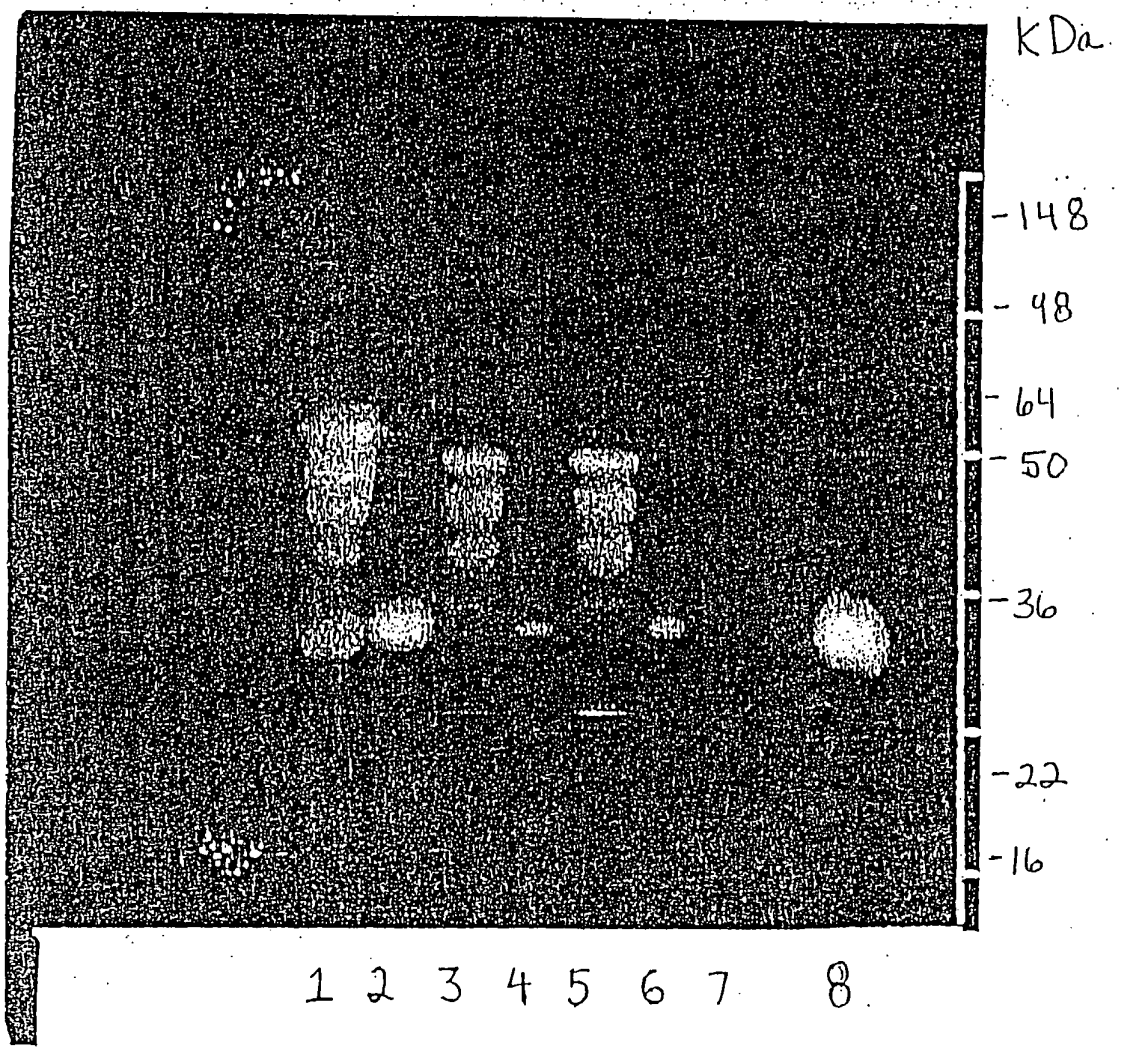
-CMVI uses ampicillin resistance.

-CMV promoter/enhancer + Intron A: nt #'s 1-1566.
-Polylinker: nt #'s 1566-1597 (5'- Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').
-SV40 polyA addition sequence: nt #'s 1598-1745.
-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

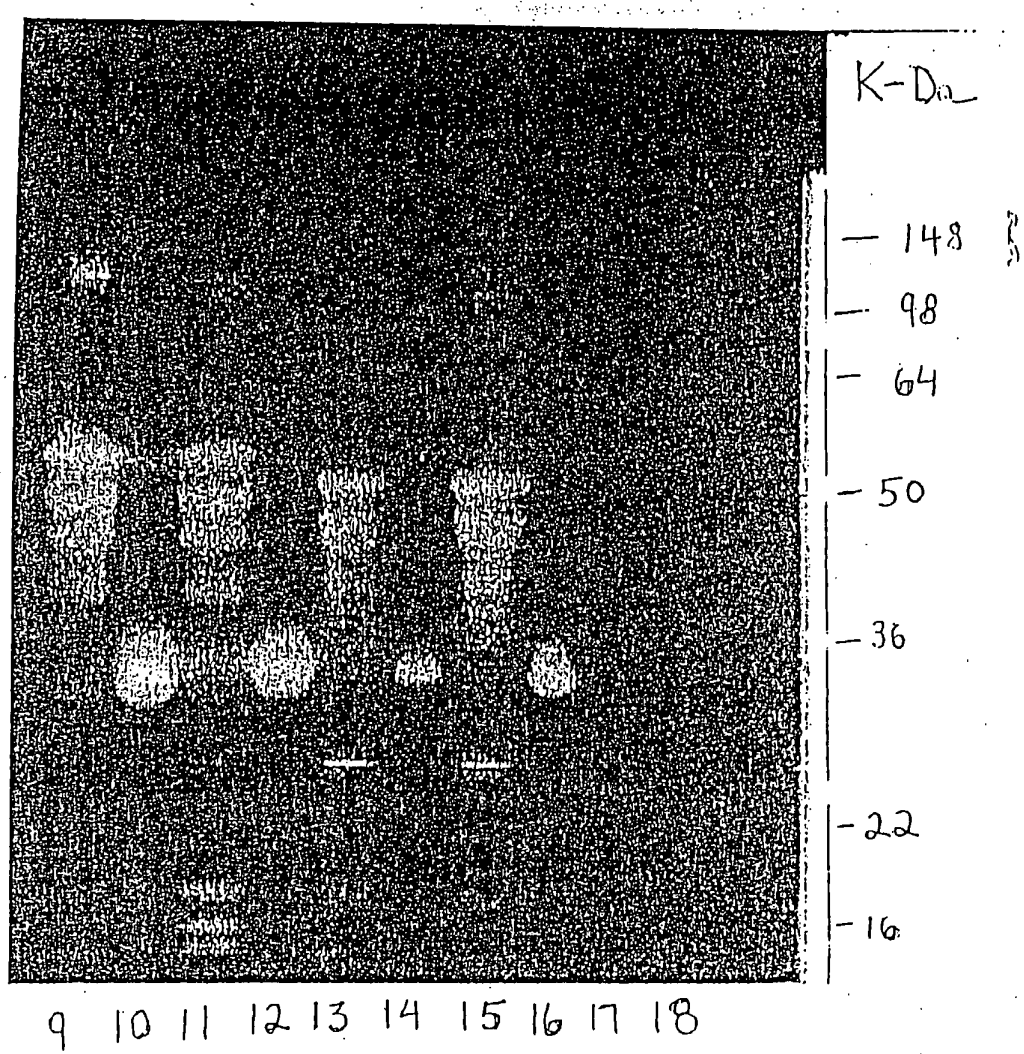
GROWTH REQUIREMENTS:



Appendix II



Power Declaration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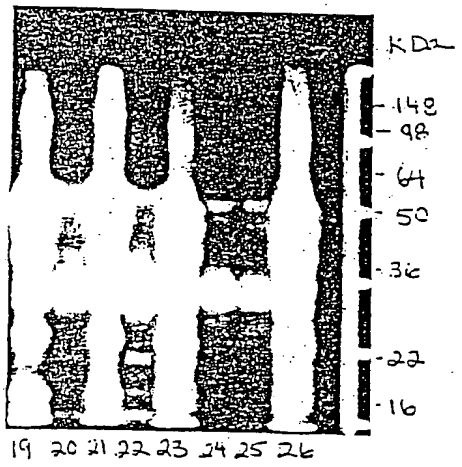

Power Declaration
Figure 1 - Gel 3

Initial Review
BOX AF

sponse Under
37 CFR 1.116- Expedited
Procedure Examining
Group 1814

PATENT
28967/32863

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Alitalo *et al.*

Serial No: 08/510,133

Filed: August 1, 1995

Title: RECEPTOR LIGAND

Group Art Unit: 1814

Examiner: Lathrop, B.

EXPRESS MAIL LABEL NO:
EM099827086US
Date of Deposit: June 11, 1997

I hereby certify that this paper is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

Mark Bonadonna

AMENDMENT AFTER FINAL ACTION

and

CONDITIONAL PETITION TO REVERSE OR WITHDRAWN ADVERSE PRIORITY DETERMINATION PURSUANT TO 37 C.F.R. §1.181

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In an official action mailed April 11, 1997, the examiner finally rejected claims 1, 8, 9, 13-15, and 19-25 variously under 35 U.S.C. §§ 101 and 112, first paragraph. Claims 2 and 12 were allowed, and claims 16 and 17 were objected to as being dependent upon a rejected base claim, but were otherwise deemed allowable. The applicants respectfully request reconsideration in light of the following amendments and remarks.

AMENDMENTS

In the specification:

At page 24, line 30, after "Figure 9" please insert -- (SEQ ID NOs: 32 and 33) --.

In the claims:

Please cancel claims 3-7 and 11, without prejudice, amend claims 8, 16, 17, 21, 22, and 25, and add new claims 26-28, as shown below.

C1

8. (Three times amended) A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding.

C2

16. (Amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, [according to claim 13,] wherein amino terminal amino acids 2 through 18 of said polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

17. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, [according to claim 1,] said polypeptide being purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

C3

21. (Amended) A polypeptide according to claim [8] 17 further comprising a detectable label.

C3 ord.

22. (Amended) A pharmaceutical composition comprising a polypeptide according to claim [8] 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

C4

25. (Amended) A pharmaceutical composition comprising a polypeptide according to claim [14] 16 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

-- 26. A polypeptide according to claim 8 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.

C5

27. A polypeptide according to claim 8 wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

28. A polypeptide according to claim 16 further comprising a detectable label. --

REMARKS

I. History of claims and explanation of amendments.

The application as filed contained twelve claims. In a preliminary amendment (Paper No. 10) dated August 12, 1996, claims 1-4, 6, 8-9, and 12 were amended, and claims 13-19 were added to the application. In an amendment dated February 10, 1997, the applicants canceled claims 10 and 18, amended claims 1, 8, 9, 14, and 17, and added new claims 20-25. In the present amendment, the applicants cancel claims 3-7 and 11, amend claims 8, 16, 17, 21, 22, and 25, and add new claims 26-28. Thus, upon entry of the foregoing amendments, claims 1-2, 8-9, 12-17, and 19-28 would be pending. A copy of the claims in their amended forms is appended hereto.

The nature of each claim amendment is discussed below in the remarks pertaining to each claim.

New claim 26 depends from claim 8 and further limits claim 8 by adopting a suggestion of the examiner with respect to subject matter that the specification enables. Support for the limitation "within amino acids 1-180 of SEQ ID NO: 33" is found in the specification at p. 28, lines 1-3. New claim 27 further limits claim 26 by reciting a specific amino terminal amino acid residue. The particular amino terminus that is recited in claim 27 corresponds to the amino terminus recited in claim 16. This amino terminus finds written support at p. 19, lines 17-19 of the specification.

II. Restriction Requirement

The applicants have canceled non-elected claims 3-7 and 11 without prejudice.

III. **The Applicants respectfully request issuance of an advisory action wherein the Patent Office reverses as incorrect, or withdraws as inappropriate, its determination that no claims in the present application are afforded priority to U.S.S.N. 08/340,011.**

In the outstanding official action, the examiner has asserted, for the first time, that no claims in the present application are entitled to priority based upon U.S.S.N. 08/340,011, filed November 14, 1994, *because of an asserted lack of written description* under 35 U.S.C. §112, first paragraph.[1] For the reasons set forth below, this determination is legally and factually incorrect. Moreover, the right of priority has no bearing on the patentability of any claim at

---

[1] In its first official action, the examiner made an initial determination that no claims were afforded priority by the '011 application, because of an *asserted absence of enabling disclosure*. However, that initial determination was made without any consideration of the preliminary amendment portion of U.S.S.N. 08/340,011 (a significant omission, since the '011 application is a Rule 62 continuation-in-part of an earlier application, and the preliminary amendment portion of the '011 application is highly pertinent to the priority issue). In the outstanding final action, the priority determination based on lack of enablement has properly been withdrawn. However, the examiner has, for the first time, raised a new objection to the priority claim, based upon an asserted lack of written description.

this time, and therefore, is an inappropriate subject for Patent Office determination.

A. The applicants respectfully request entry into the record and consideration of the expert declaration of Dr. Carl-Henrik Heldin filed herewith.

The Patent Office's reviewing court has explicitly approved of the use of declarations which offer factual evidence to help resolve the issue of "written description" in a patent application, and has held that failure to accord appropriate weight to such declarations constitutes legal error. See *In re Alton*, 37 U.S.P.Q. 1578, 1583 (Fed. Cir. 1996). The applicants have filed herewith the expert declaration of Dr. Carl-Henrik Heldin (the "Heldin declaration") to offer a factual explanation as to *why* one of ordinary skill in the art would have understood the 1994 priority application to describe the invention presently being claimed. Since the examiner raised the written description issue for the first time in the outstanding final official action,[2] the applicants respectfully request entry of this declaration into the record and consideration thereof with respect to the issue of written description.

B. The determination that no claims are entitled to priority is legally and factually incorrect.

The law is clear that original claims (i.e., claims contained in the patent application as filed) comply with the written description requirement of §112, because *original claims constitute their own description*. See *In re Koller*, 204 U.S.P.Q. 702, 706 (C.C.P.A. 1980). Moreover, later added claims of similar scope and wording are described by original claims. *Id.*

In the present case, the applicants' 1994 priority application (the '011 application) contained original claims to an Flt4 ligand. For example, original claim 31 recites, "A ligand which specifically binds to an FLT-4 receptor

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[2] The written description issue was <u>not</u> necessitated solely by amendments made by the applicants in response to the first action on the merits, and therefore could have been raised by the Patent Office prior to the issuance of a final action.

tyrosine kinase." By way of comparison, claim 1 of the present application recites, "A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase."

Claim 1 is unquestionably of similar scope and wording to claim 31 as originally filed. Whereas original claim 31 was directed to "a ligand," claim 1 is directed to "a purified and isolated polypeptide." However, the 1994 priority application clearly states that the ligand of the invention is a purified protein. (See, e.g., preliminary amendment to U.S.S.N. 08/340,011 dated November 14, 1994, at p. 15: "The purified biologically active ligand protein . . . .") Whereas original claim 31 was directed to binding to Flt4 receptor tyrosine kinase, claim 1 clarifies that the ligand binds to *the extracellular domain* of Flt4. However, the 1994 priority application clearly states that the ligand protein binds to the Flt4 *extracellular domain*. (See, e.g., preliminary amendment to U.S.S.N. 08/340,011 dated November 14, 1994, at p. 11: "The above experiments prove that the ligand binds to the recombinant FLT4 EC domain.") Finally, original claim 31 recites that the ligand "specifically binds" whereas claim 1 is directed to "high affinity" binding. However, this difference merely adopts preferred claim language suggested by the examiner in the course of prosecution. Thus, claim 1 is unquestionably of similar scope and wording to an original claim of the '011 patent application. (See the Heldin declaration at ¶ 6.) Accordingly, original claims in the '011 patent application provide written description support for claim 1 of the present patent application. See *In re Koller*, 204 U.S.P.Q. at 706.

Claim 19, which depends from claim 1 and recites that the polypeptide further comprises a detectable label, finds written description support in original claim 33 of the 1994 priority application. See *In re Koller*, 204 U.S.P.Q. at 706; see also the Heldin declaration at ¶ 7.

Claim 17 (which depended from claim 1 but has been rewritten as an independent claim incorporating the limitations of claim 1) is similar to claim 1 and additionally recites that the polypeptide is purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, the cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity

purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase. These additional limitations find explicit written description support in the 1994 priority application in Examples 12 and 15: Example 12 teaches that conditioned media from the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) produces a soluble ligand for Flt4 that binds to recombinant Flt4 extracellular domain and that can be purified using the Flt4 EC domain in affinity chromatography; Example 15 describes such affinity chromatography. (See the preliminary amendment to the '011 application at pp. 8-11 and 15.) Thus, claim 17 finds written description support in the original claims of the 1994 priority application coupled with the written description provided in Examples 12 and 15. (See the Heldin declaration at ¶ 8.)

Claim 21 as amended is identical to claim 19 except that claim 21 depends from claim 17. Thus, claim 21 finds written description support in the 1994 priority application for the reasons outlined above with respect to claims 17 and 19.

Claim 14 recites "A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase." Written description support for "a purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase" is discussed above in relation to claim 1. Example 12 in the 1994 priority application teaches that the Flt4 polypeptide ligand stimulates Flt4 tyrosine phosphorylation in mammalian cells that express Flt4 receptor tyrosine kinase. (See the preliminary amendment to the '011 application at pp. 8-11; see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or fragment thereof, which is capable of stimulating the Flt4 receptor . . . .") Thus, claim 14 finds written description support in the original claims of the '011 application coupled with the written description provided in Example 12. (See the Heldin declaration at ¶ 9.)

Claims 13 depends from claim 1 and recites that the polypeptide has an apparent molecular weight of approximately 23 kD as assessed by SDS-

PAGE under reducing conditions. The approximate 23 kD molecular weight is an *inherent property* of the Flt4 ligand that the '011 application teaches one how to purify from the PC-3 conditioned medium. (See the Heldin declaration at ¶¶ 10 and 11.A.) As such, the inclusion of this property in the present application and in claim 13 does not deprive claim 13 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*, 33 U.S.P.Q.2d 1274, 1276 (Fed. Cir. 1995) ("A claim in a CIP application is entitled to the filing date of the parent application when the claimed invention is described in the parent specification in a manner that satisfies, inter alia, the description requirement of 35 U.S.C. §112. . . . [T]he later explicit description of an inherent property does not deprive the product of the benefit of the filing date of the earlier application."); *Ex parte Yamaguchi*, 6 U.S.P.Q.2d 1805, 1807 (PTO Bd. App. 1987) (Claim to compound characterized by a particular x-ray diffraction spectrum has written description support in earlier application that teaches the compound, notwithstanding the absence of any teaching of the x-ray diffraction pattern in the earlier application, because a compound and all of its properties are inseparable); see also *Application of Davies*, 177 U.S.P.Q. 381, 385, 475 F.2d 667, 671-72 (C.C.P.A. 1973) ("[W]e see no impediment to the present applicants' refiling their application and incorporating a discussion of the allegedly unobvious properties [of the invention] while retaining the effective date of the application involved here through §120.")

Claim 15 depends from claim 14 and further recites that the polypeptide "comprises an amino acid sequence set forth in SEQ ID NO: 13." This partial amino acid sequence is an *inherent property* of an Flt4 ligand that the 1994 priority application teaches one how to purify from the PC-3 conditioned medium. (See the present application at p. 19, lines 9-19 (teaching that Flt4 ligand affinity purified from PC-3 medium has an amino terminal amino acid sequence set forth in SEQ ID NO: 13); see also the Heldin declaration at ¶¶ 10 and 11.B.) As such, the inclusion of this property in the present application and in claim 15 does not deprive claim 15 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies*, *supra*.

Claim 16 (which depended from claim 13 but has been rewritten in independent form) additionally recites amino terminal amino acid sequence information of the claimed polypeptide. The recited amino acid sequence is an *inherent property* of the Flt4 ligand that the 1994 priority application teaches one how to purify from PC-3 conditioned medium.[3] (See the Heldin declaration at ¶¶ 10 and 11.C.) As such, the inclusion of this property in the present application and in claim 16 does not deprive claim 16 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies*, *supra*.

New claim 28 is identical to claim 19 except that claim 28 depends from claim 16. Thus, claim 28 finds written description support in the 1994 priority application for the reasons outlined above with respect to claims 16 and 19.

Claim 23 depends from claim 14 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." This molecular weight limitation is an *inherent property* of an Flt4 ligand that the 1994 priority application teaches one how to purify from the PC-3 conditioned medium, as discussed above with respect to claim 13. (See also the Heldin declaration at ¶¶ 10 and 11.D.) As such, the inclusion of this property in the present application and in claim 23 does not deprive claim 23 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies*, *supra*.

Moreover, the foregoing is not intended to be a complete list of those claims which find written description support in the specification. See the Heldin declaration at ¶11.)

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[3] Moreover, the 1994 priority application teaches to determine the amino terminal amino acid sequence. (See preliminary amendment to '011 application at p. 15 (Example 15).)

C. The Patent Office's reliance on the *Fiers* case is improper.

In dismissing the applicants' priority claim on written description grounds, the Patent Office relied upon the Federal Circuit's decision in *Fiers v. Revel*, 25 U.S.P.Q.2d 1601, 1606 (Fed. Cir. 1993). (Official action at pp. 2 and 3.) However, the *Fiers* opinion was rendered on its own distinct set of facts, and was rendered in the context of the state of the art in 1979-81 (i.e., about 13-15 years prior to the applicants' 1994 filing date). Since the issue of written description is factual in nature, *In re Alton*, *supra*, 37 U.S.P.Q.2d at 1580, the examiner's reliance upon a legal opinion rendered on different facts, and in a much earlier period of the art of molecular biology, is highly suspect from the outset.

1. The present application is distinguishable from the facts of the *Fiers* case because the present application teaches a method of preparing the claimed protein as a natural isolate.

The *Fiers* opinion was based on the premise that a written description of a DNA invention requires the same degree of specificity as a conception of a DNA invention. *Fiers*, 25 U.S.P.Q.2d at 1606. Citing its earlier opinion in *Amgen v. Chugai Pharmaceutical Co.*, 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991), the Court acknowledged that conception of a DNA can occur where one is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. *Fiers*, 25 U.S.P.Q.2d at 1604. In the present case, the 1994 priority application is able to define the Flt4 ligand protein by a method of preparation (e.g., affinity purification using the Flt4 extracellular domain) and by chemical characteristics (e.g., a polypeptide that is capable of stimulating the Flt4 receptor and regulating vascular endothelial cells). Thus, under the standards articulated in the *Fiers* and *Amgen* cases for DNA inventions, the 1994 priority application contains a written description of the Flt4 ligand protein invention claimed herein.

2. The present application is distinguishable from *Fiers* because the invention presently claimed pertains to a purified protein.

In *Fiers*, the Federal Circuit rendered an opinion as to that which is required under §112, first paragraph, for an adequate written description of a DNA invention. The invention claimed in the present application is not a DNA invention;[4] the invention pertains to a purified protein, and the issue concerns whether a priority application contains a sufficient written description of that protein invention. The examiner has failed to articulate why a factual determination in *Fiers* pertaining to a DNA invention is relevant to a factual determination pertaining to a protein invention in the present case.[5] Accordingly, the examiner has failed to meet his burden of establishing a *prima facie* case of lack of written description.

Since the *Fiers* holding is distinguishable on its facts and also was rendered in the context of the state of the art in 1979-81, i.e., about 13-15 years prior to the applicants' 1994 filing date, the *Fiers* opinion fails to support the examiner's written description objection.

D. The right of priority has no bearing on the patentability of any claim at this time, and therefore, is an inappropriate subject for Patent Office determination.

The Manual of Patent Examining Procedure instructs that a priority determination should be made during *ex parte* prosecution *only* when an intervening reference is found, upon which a rejection under §102 or §103 would be made:

> The only times during *ex parte* prosecution that the examiner considers the merits of an applicant's claim of

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[4] The Patent Office has deemed the DNAs taught in the application to constitute a distinct invention.

[5] In this regard, the Patent Office's attention is directed to *Scripps Clinic v. Genentech Inc.*, 18 U.S.P.Q. 1001 (Fed, Cir. 1991), an opinion issued contemporaneously with the *Amgen* opinion and pertaining to a purified protein invention. The Patent at issue in the *Scripps* case (Reissue Patent No. 32,011) contained claims to a purified protein (Factor VIII:C) and to an affinity method of purifying the protein. No amino acid sequence description was required under §112, first paragraph, for the Patent Office to issue or to reissue this patent.

> priority is when a reference is found with an effective date between the date of the foreign filing and the date of filing in the United States and when an interference situation is under consideration. If at the time of making an action the examiner has found such an intervening reference, he or she simply rejects whatever claims may be considered unpatentable thereover, without paying any attention to the priority date . . . .

(M.P.E.P.((6th Ed., Rev. 2) §201.15.)

The outstanding final action constitutes the first time that the Patent Office has raised its written description objection as a basis for refusing to afford priority to the '011 application.[6] However, there are no prior art rejections based upon intervening references in the outstanding action. Accordingly, under the Patent Office's own procedures, it was inappropriate to consider the merits of the priority claim in the official action.

E. Conditional Petition to Reverse or Withdrawn Adverse Priority Determination.

Should the examiner refuse to reverse or withdraw the adverse priority determination that was made for the first time in the final official action, the applicants hereby petition the commissioner to reverse this determination as improper, or, in the alternative, to withdraw this determination as premature and expunge from the file all mention of this premature determination. The facts in support of reversal of the priority determination are provided in parts A-C, above, and in the Declaration of Dr. Heldin filed herewith. The facts in support of withdrawal of the premature determination are provided in part D, above. In the event of withdrawal, the applicants respectfully submit that all mention of the priority determination in the final official action and this submission by the applicants should be expunged from the file, so as not to taint the file history of the eventual patent in a manner adverse to the applicants.

The priority issue is properly the subject of a petition because the priority determination is not pertinent to any rejection and, therefore, is not

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[6] See note 1, *supra*.

subject to review by the Board of Patent Appeals and Interferences. See M.P.E.P. §706.01.

The applicants hereby authorize the commissioner to charge any necessary petition fee associated with this conditional petition to Deposit Account No. 13-2855. This petition has been timely filed within two months of the mailing of the final official action that contains the adverse priority determination at issue.

IV. **The amendments to claim 8 render moot the rejection of claims 8-9 and 19-20.**

In paragraph 9 of the outstanding official action, the examiner rejected claims 8-9 and 20-22 under 35 U.S.C. § 101, asserting that these claims read on a product of nature, because claim 8 fails to recite a "purified and isolated" polypeptide. (Office action at p. 5.)

In response, the applicants have amended claim 8 to recite, "A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding." Thus, amended claim 8 does not read on a product of nature, rendering the rejection of claim 8 (and claims 9 and 19-20 which depend therefrom) moot. Since this amendment adopts a suggestion of the Patent Office and removes an issue for appeal, entry of the amendment and withdrawal of the rejection is respectfully requested.

V. **The amendments to claims 16 and 17 place these claims in condition for allowance.**

In paragraph 15 of the outstanding action, the Patent Office objected to claims 16 and 17 as being dependent upon a rejected base claim, but indicated that these claims would be allowable if rewritten in independent form. (Office action at p. 11.) In response, the applicants have rewritten claims 16 and 17 in independent form, incorporating all of the limitation of the base claim and any intervening claims. Accordingly, claim 16 and 17 are now in condition for allowance.

VI. The amendments to claims 21, 22, and 25 place these claims in condition for allowance; and new claim 28 is in condition for allowance.

Claims 21, 22, and 25 have been amended to depend from and further limit claims 16 and 17. New claim 28 is identical to claim 21 and depends from claim 16. Because the subject matter of claims 16 and 17 has been deemed allowable, the amendment of claims 21, 22, and 25 (and addition of claim 28) to depend from claims 16 and 17 also places these claims in condition for allowance. Accordingly, entry of these amendments and allowance of claims 21, 22, 25, and 28 is respectfully requested.

VII. The Patent Office's rejections of clams 1, 8, 9, 13-15, and 19-25 under §112, first paragraph, for lack of enablement improperly ignore both guidance provided in the specification and the skill of those in the art.

In paragraphs 10-13 of the official action, the examiner articulated his basis for maintaining rejections of claims 1, 8, 9, 13-15, and 19-25 under §112, first paragraph, for lack of enablement. The Patent Office admits that fragments of the protein of SEQ ID NO: 33 can be made, but asserts that undue experimentation would be required to screen all fragments of SEQ ID NO: 33 to determine which fragments bind the receptor:

> The examiner argues that the claim limitation of binding the receptor must be met by testing all fragments encompassed by the claims, which in this case are not limited in any way.

(official action at p. 7.)[7]

The Patent Office's insistence that it is necessary to test all fragments of SEQ ID NO: 33 ignores the scientific ability of one of ordinary skill in the art. Importantly, one of ordinary skill in the art would not conduct experimentation by haphazardly making <u>all</u> of the possible fragments of SEQ ID NO: 33 and testing their ability to bind the receptor. An artisan of ordinary skill understands that each fragment that is screened provides guidance as to that

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[7] Claim 8 encompasses only polypeptides which are capable of binding the Flt4 receptor. To the extent that the examiner has interpreted claim 8 (or similarly limited claims) to "encompass" all fragments of SEQ ID NO: 33, the examiner has ignored a limitation of claim 8 and thereby erroneously construed the claim.

portion of SEQ ID NO: 33 that is effective for binding, and that portion which is not.[8] An artisan of ordinary skill also understands techniques for accelerating a screening process,[9] and techniques for screening multiple polypeptides *simultaneously*. Thus, the examiner's reasoning greatly overstates both the quantity and the nature of the experimentation required to practice the invention as claimed.

In this regard, the application provides explicit guidance for screening fragments of SEQ ID NO: 33 to determine a portion effective to permit Flt4 binding. Although SEQ ID NO: 33 contains 350 amino acids, the specification provides guidance that the region critical for receptor activation is contained within its first approximately 180 amino acid residues.

> By extrapolation from studies of the structure of the related platelet derived growth factor (PDGF, reference Heldin, et al., Growth Factors 8, 245-252, 1993) one determines that the region critical for receptor activation by the Flt4 ligand is contained within its first approximately 180 amino acid residues. (Specification, pp. 27-28.)

To determine which fragments contain a sufficient portion of SEQ ID NO: 33 to permit binding, the specification also outlines a specific protocol. The specification teaches one skilled in the art to (a) generate progressive deletion products of the Flt4 ligand cDNA; (b) express these modified cDNAs; and (c) assay the resulting truncated protein forms, e.g., by studying their ability to induce Flt4 autophosphorylation. (Specification at, e.g., p. 27, lines 23-29.) These teachings serve to both provide guidance for predicting the portions of

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[8] For example, a determination that a polypeptide comprising residues 34-180 of SEQ ID NO: 33 is effective to permit binding to Flt4 and that a polypeptide comprising residues 181-350 is ineffective to permit binding would provide significant guidance as to that portion of SEQ ID NO: 33 to further screen for effective fragments. Thus, the assertion that it would be necessary to screen "all" fragments of SEQ ID NO: 33 to practice the claimed invention relies upon the false assumption that individual screening assays will be performed without knowledge gained from prior screenings.

[9] For example, it is within the skill of the art to synthesize spaced deletion mutants (e.g., residues 34-350, 34-330, 34-310, etc.) from SEQ ID NO: 33, rather than successive deletion mutants (34-350, 34-349, 34-348 . . .), to more rapidly identify effective portions for binding Flt4.

SEQ ID NO: 33 that are effective to permit Flt4 binding; and (2) reduce the amount of experimentation required to determine the minimum portion of SEQ ID NO: 33 that is critical for receptor binding.

Moreover, as explained above, it is within the skill of the art to synthesize deletion mutants of SEQ ID NO: 33 that have been spaced intermittently (e.g., residues 34-180, 34-160, 34-140, 34-120, etc.), rather than synthesize every possible successive deletion mutant (34-180, 34-179, 34-178, 34-177 . . .), to more rapidly identify effective portions for binding Flt4. Furthermore, the skilled artisan is capable of synthesizing and screening several such deletion fragments simultaneously, in parallel experiments. Thus, the examiner's assertions that it is necessary to screen every fragment of SEQ ID NO: 33, that the specification lacks guidance, and that the amount of screening required constitutes undue experimentation is improper. See *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988) ("Enablement is not precluded by the necessity for some experimentation such as routine screening. . . . The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.")

VIII. Summary

For the foregoing reasons, the applicants respectfully request reconsideration, withdrawal of all claim rejections and objections to the specification, withdrawal of the notation that no claims are afforded priority to the parent application, and allowance of claims 1-2, 8-9, 12-17, and 19-28.

Respectfully submitted,

June 11, 1997

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Appendix of claims

1. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase.

2. (Amended) A purified and isolated polypeptide comprising an amino acid sequence shown in SEQ ID NO: 33.

8. (Three times amended) A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding.

9. (Twice amended) A polypeptide according to claim 8 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

12. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 2 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

13. A polypeptide according to claim 1 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

14. (Amended) A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

15. A purified and isolated polypeptide according to claim 14, said polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 13.

16. (Amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, wherein amino terminal amino acids 2 through 18 of said polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

17. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, said polypeptide being purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

19. A polypeptide according to claim 1 further comprising a detectable label.

20. A polypeptide according to claim 8 which is capable of binding the extracellular domain of Flt4 receptor tyrosine kinase with high affinity and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

21. (Amended) A polypeptide according to claim 17 further comprising a detectable label.

22. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

23. A polypeptide according to claim 14 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

24. A polypeptide according to claim 14 comprising a portion of SEQ ID NO: 33 effective to permit binding to Flt4 receptor tyrosine kinase and stimulation of Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

25. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 16 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

26. A polypeptide according to claim 8 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.

27. A polypeptide according to claim 8 wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

28. A polypeptide according to claim 16 further comprising a detectable label.

PATENT
28967/32863

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

Alitalo et al.

Serial No.: 08/510,133

Filed: August 1, 1995

For: RECEPTOR LIGAND

Group Art Unit: 1814

Examiner: Lathrop, B.

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I hereby certify that this paper and the documents referred to as enclosed herewith are being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Mark Bonadonna

Declaration of Carl-Henrik Heldin
Pursuant to 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Carl-Henrik Heldin, hereby state as follows:

1. I am Director and member of the Uppsala Branch of Growth Regulation of the Ludwig Institute of Cancer Research (the Ludwig Institute) in Uppsala, Sweden. My curriculum vitae is attached hereto as Exhibit A.

2. I understand that on 01 August 1995, Dr. Kari Alitalo and Dr. Vladimir Joukov (as inventors) filed U.S. Patent Application Serial No. 08/510,133 (hereinafter "the 1995 application"), directed to a polypeptide ligand for Flt4 receptor tyrosine kinase; fragments thereof; a polynucleotide encoding the ligand; vectors and host cells comprising the polynucleotide; and

antibodies reactive with the ligand. I understand that the Ludwig Institute now has an ownership interest in this application.

3. I further understand that, during examination of the 1995 application by the U.S. Patent and Trademark Office (the Patent Office), the examiner has taken the position that U.S. Patent Application Serial No. 08/340,011, filed on 14 November 1994 ("the 1994 application") does not contain a written description of the polypeptide invention that is being claimed in the 1995 application. I have been asked by the Ludwig Institute to review the 1994 and 1995 applications and to provide a factual analysis of whether the 1994 application contains a written description of the invention that is being claimed in the 1995 application.

4. I understand that the claims in a patent application are the portion of a patent application that defines the invention for which patent applicants seek patent protection. I further understand that patent applications are written for the practitioner of ordinary skill in the pertinent scientific field. In the scientific specialties or subdisciplines which fall within the general category of "cellular and molecular biology," the reader of ordinary skill in 1994 and 1995 (hereinafter "the reader") would have had at least a medical or doctorate degree and probably at least some post-doctoral research experience.

5. To perform this analysis, I have reviewed and understand the contents of the 1994 application. This review included the document titled "Preliminary Amendment" that was filed on 14 November 1994 (hereinafter "the Preliminary Amendment"). I understand that pages 2-19 of the Preliminary Amendment contain text, examples, and claims which are considered part of the 1994 application. I also have reviewed and understand the contents of the 1995 application, including the claims thereof. Exhibit B hereto contains the pending claims of the 1995 application, with claim amendments that the Applicants intend to file with the Patent Office contemporaneously with this declaration.

6. From the facts summarized below, I conclude that the subject matter of claim 1 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Stated another way, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 1 of the 1995 application, at the time that the 1994 application was filed:

A. Claim 31 of the 1994 application recites, "A ligand which specifically binds *to an FLT-4 receptor tyrosine kinase*." (See the Preliminary Amendment at p. 18.) Since claims in a patent application define the invention for which patent applicants seek patent protection, it is absolutely clear to me that the inventors considered an Flt4 ligand to be an aspect of their invention.

B. Claim 1 of the 1995 application recites, "A purified and isolated polypeptide capable of binding with high affinity *to the extracellular domain of Flt4 receptor tyrosine kinase*." Thus, whereas claim 31 of the 1994 application was directed to "a ligand," claim 1 of the 1995 application is directed to "a purified and isolated polypeptide." However, the 1994 application clearly states that the ligand of the invention is a purified protein. (See, e.g., the Preliminary Amendment at p. 15 ("The purified biologically active ligand protein . . . ."); see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or a fragment thereof, which is capable of stimulating the FLT4 receptor....").) Therefore, the "purified and isolated polypeptide" recitations of claim 1 are described in the 1994 application.

C. Whereas claim 31 of the 1994 application was directed to binding "to an FLT-4 receptor tyrosine kinase," claim 1 of the 1995 application specifies that the ligand binds "to *the extracellular domain* of Flt4 receptor tyrosine kinase." However, the 1994 application clearly states that the ligand protein binds to the Flt4 *extracellular domain*. (See, e.g., the Preliminary Amendment at p. 11: "The above experiments prove that the ligand binds to the recombinant FLT4 EC [extracellular] domain.")

Therefore, the recitations in claim 1 regarding binding to the Flt4 *extracellular domain* are described in the 1994 application.

D. Claim 31 of the 1994 application recites that the ligand "specifically binds," whereas claim 1 of the 1995 application is directed to "high affinity" binding. However, the reader would have understood that the "ligand" that "specifically binds" to Flt4 receptor was a high affinity binding partner. For example, the teaching in the 1994 application to purify the ligand using the recombinant FLT4 EC domain in affinity chromatography (see, e.g., the Preliminary Amendment at p. 11 and Example 15) apprises the reader that the ligand is thought to be a high affinity ligand.

Thus, I conclude that the subject matter of claim 1 of the 1995 application is described in claim 31, at pp. 11 and 15 of the Preliminary Amendment, and elsewhere in the 1994 application.

7. I conclude that the subject matter of claim 19 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 19 of the 1995 application is directed to the polypeptide having all of the features recited in claim 1 of the 1995 application, and "further comprising a detectable label." Thus, the only aspect of claim 19 not already discussed above (in paragraph 6) is the inclusion of a detectable label. However, claim 33 of the 1994 patent application recites, "The ligand according to claim 31 comprising a label." (See the Preliminary Amendment at p. 18.) Since claims in a patent application define the invention for which patent applicants seek patent protection, it is absolutely clear to the reader from claims 31 and 33 of the 1994 application that the inventors considered an Flt4 ligand which includes a label to be an aspect of their invention. The property of being "detectable" is understood in the art to be inherent in a "label." (The purpose of a label is to provide a means for detecting the substance that carries the label.) Moreover, this understanding is confirmed by claims 34 and 35 of the 1994 application, which are directed to methods which involve "detecting" the labeled ligand.

(See the Preliminary Amendment at p. 19.) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 19 of the 1995 application, at the time that the 1994 application was filed.

8. I conclude that the subject matter of claim 17 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 17 is similar to claim 1 of the 1995 application and additionally recites that the polypeptide is "purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase." These additional properties are explicitly described in the 1994 application in Examples 12 and 15: Example 12 teaches that conditioned media from the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) produces a soluble ligand for Flt4 that binds to recombinant Flt4 extracellular domain and that can be purified using the Flt4 EC domain in affinity chromatography (see the Preliminary Amendment at pp. 8-11); Example 15 describes such affinity chromatography. (*Id.* at p. 15.) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 17 of the 1995 application, at the time that the 1994 application was filed.

9. I conclude that the subject matter of claim 14 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 14 recites, "A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase." Descriptive support in the 1994 application for "a purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase" is discussed above with respect to claim 1. (See paragraph 6, above.) Example 12 in the 1994 application teaches that the

Flt4 polypeptide ligand stimulates Flt4 tyrosine phosphorylation in mammalian cells that express Flt4 receptor tyrosine kinase. (See the Preliminary Amendment at pp. 8-11; see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or fragment thereof, which is capable of stimulating the Flt4 receptor....").) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 14 of the 1995 application, at the time that the 1994 application was filed.

10. It is fundamental biochemistry that polypeptides are organic chemical compounds, albeit sometimes large and complex ones. Like all organic chemical compounds, polypeptides may be characterized by any of several inherent physical properties, such as molecular formula and molecular weight. Such physical properties are *inherent* characteristics of organic molecules in that they are intrinsic properties of the molecules. Because polypeptides are themselves composed of covalently-bonded chains of smaller organic moieties called amino acids (of which there are about 20 naturally occurring), it is conventional to express the molecular formula of polypeptides as an amino acid sequence. The amino acid sequence of any polypeptide is an inherent property of that polypeptide.

11. Certain claims in the 1995 application recite subject matter that is described in the 1994 application, and also recite certain inherent properties of that subject matter.

A. For example, claims 13 recites a polypeptide having all of the characteristics described in claim 1 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." The subject matter of claim 1 is described in the 1994 application. (See paragraph 6, above.) The approximate 23 kD molecular weight that is recited in claim 13 is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from the PC-3 conditioned medium. (See the

1995 application at pp. 18-19 (teaching that the Flt4 ligand that was affinity purified from PC-3 medium had an apparent molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions).)

B. Claim 15 recites a polypeptide having all of the characteristics described in claim 14 and further recites that the polypeptide comprises "an amino acid sequence set forth in SEQ ID NO: 13." The partial amino acid sequence set forth in SEQ ID NO: 13 of the 1995 application is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from the PC-3 conditioned medium. (See the 1995 application at p. 19, lines 9-19 (teaching that Flt4 ligand that was affinity purified from PC-3 medium had an amino terminal amino acid sequence set forth in SEQ ID NO: 13).)

C. Claim 16 recites a polypeptide having all of the characteristics described in claim 13 and further recites that amino acids 2 through 18 of the polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13. Thus, for the reasons described above with respect to claims 13 and 15 (in Parts A and B), the features recited in claim 16 are inherent properties of an Flt4 ligand that the 1994 application teaches one how to purify from PC-3 conditioned medium.

D. Claim 23 recites a polypeptide having all of the characteristics described in claim 14 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." The subject matter of claim 14 is described in the 1994 application. (See paragraph 9, above.) The approximate 23 kD molecular weight further recited in claim 23 is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from a PC-3 conditioned medium, as discussed in Part A above with respect to claim 13.

The foregoing is not intended to constitute a complete list of those claims which recite inherent properties of an Flt4 ligand described in the 1994 application. For example, the 1995 application teaches a cDNA nucleotide sequence and a

deduced amino acid sequence of a precursor of a 23 kD Flt4 ligand taught in the 1994 application. (See, e.g., 1995 application at p. 5, lines 13-20.) Thus, according to the 1995 application, an inherent property of an Flt4 ligand taught in the 1994 application is that the ligand has an amino acid sequence comprising a portion of SEQ ID NO: 33 that is effective to permit binding to Flt4 receptor tyrosine kinase and stimulate phosphorylation thereof. These properties are recited in several claims of the 1995 application other than those specifically discussed above.

12. The 1994 application teaches the reader how to purify and isolate an Flt4 ligand from conditioned medium of a prostatic cell line, using an affinity chromatography method:

A. Example 12 in the 1994 application teaches the reader how to prepare a conditioned medium comprising an Flt4 ligand by culturing the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) for seven days in F12 medium in the absence of serum, and then clarifying the medium by centrifugation. (See the Preliminary amendment at p. 8.) Example 4 in the 1995 application contains a similar teaching.

B. Example 12 in the 1994 application contains experimental data proving that the PC-3 conditioned medium contains a ligand that is capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase, in cells expressing Flt4 receptor tyrosine kinase. (See the Preliminary Amendment at pp. 8-11.) Moreover, Example 12 in the 1994 application characterizes the Flt4 ligand as a moiety of at least 10,000 molecular weight, and teaches that the medium can be concentrated with a commercially available Centricon-10 concentrator, in order to increase Flt4 ligand activity. (Preliminary Amendment at p. 11.)

C. Example 12 further teaches that treatment of the concentrated PC-3 conditioned medium with Flt4 extracellular domain fragment coupled to Sepharose beads (a solid support) will remove the Flt4 ligand from the conditioned medium. (See the Preliminary Amendment at p. 11 (pretreatment of the concentrated conditioned medium with Flt4EC-

Sepharose abolished the ability of the conditioned medium to stimulate Flt4 phosphorylation).) This teaching provides direct evidence that the ligand of the invention binds to the extracellular domain of Flt4, and thus that the ligand can be purified using the recombinant Flt4 extracellular domain in affinity chromatography.

D. Example 14 of the 1994 application teaches how to make recombinant Flt4 extracellular domain protein to use in an affinity chromatography matrix to purify the Flt4 ligand. (See, e.g., the Preliminary Amendment at p. 13.) Example 3 of the 1995 application contains a similar teaching.

E. Example 15 of the 1994 application teaches how to purify the Flt4 ligand using affinity chromatography procedures. In one of the procedures, the affinity matrix is Flt4 extracellular domain protein that has been cross-linked to CNBr-activated Sepharose 4B (a commercially available solid support that is useful for generating an affinity matrix). The reader in 1994 would have understood that affinity purification involves contacting the ligand-containing solution with the affinity matrix to permit binding between the ligand and the affinity matrix; washing the affinity matrix to remove unbound impurities; and eluting the ligand with an eluting solution. Typically, all fractions removed from the matrix (wash fractions and elution fractions) are assayed to determine in which fractions the ligand of interest has eluted. Example 15 of the 1994 application teaches to use an Flt4 phosphorylation assay to determine which chromatography fractions contained the Flt4 ligand. (See the Preliminary Amendment at p. 15.) The phosphate buffered saline and phosphate buffer wash solutions that were actually used (see the 1995 application at Example 5, p. 18) are typical wash solutions for a protein affinity chromatography. Moreover, the reader would have known that varying parameters such as ionic strength, pH, and the hydrophilic/hydrophobic character of the eluting solutions are conventional methods for eluting a compound of interest from an affinity chromatography column. Thus, the details in Example 15 of the 1994

application enable the reader to purify the Flt4 ligand by affinity chromatography.

F. The 1994 application teaches to subject the Flt4 ligand material that is eluted from the affinity column to further purification, using ion exchange and reverse-phase high pressure chromatography and SDS-polyacrylamide gel electrophoresis. (See the Preliminary Amendment at p. 15.) While the reader would have been able to perform all three of these conventional techniques, it is clear from the results reported in the 1995 application that sufficiently pure Flt4 ligand is obtained (e.g., sufficiently pure for amino acid sequencing) simply with the affinity purification followed by the SDS-PAGE procedure. (See the 1995 application at Example 5, pp. 17-19.) The ion exchange and reverse-phase chromatography were unnecessary.

Thus, the 1994 application teaches the reader how to purify and isolate an Flt4 ligand. The 1995 application describes results of such a purification, thereby demonstrating that the affinity purification method taught in the 1994 application works successfully.

13. The 1994 application teaches several uses for purified Flt4 ligand. These uses include:

A. Isolating a gene encoding the Flt4 ligand by microsequencing the purified ligand to determine a partial amino acid sequence; generating oligonucleotide probes based on the amino acid sequence (See the Preliminary Amendment, Example 15, p. 15; and Example 12, pp. 11-12); using the oligonucleotides as hybridization probes or PCR primers to isolate a ligand-encoding cDNA clone from a cDNA library generated from PC-3 poly-A RNA (*Id.*, Examples 16 and 17A, p. 16);

B. use in an assay system to screen for inhibitors of Flt4 ligand/Flt4 receptor tyrosine kinase interaction (Preliminary Amendment at pp. 6 and 7);

C. regulating the growth, differentiation, and functions of endothelial cells, particularly lymphatic endothelia (Preliminary Amendment at p. 7);

D. generating antibodies against the Flt4 ligand (Preliminary Amendment at p. 7);

E. use in an assay to detect the presence of FLT4 receptor tyrosine kinase (see the Preliminary Amendment at p. 19, claim 35); and

F. use in an assay to detect endothelial cell proliferation (*Id.* , claim 34).

14. With respect to my conclusions in paragraphs 6-13, above, I believe that the reader of ordinary skill in the field in 1994 who reviewed the 1994 application would have reached the same conclusions: that the inventors had possession of a concept of what is now being claimed in the present application. Stated another way, the priority application reasonably would have conveyed to the skilled artisan that the inventors had possession of the Flt4 ligand invention recited in claims of the 1995 application, of how to purify the ligand, and how to use the ligand.

15. I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

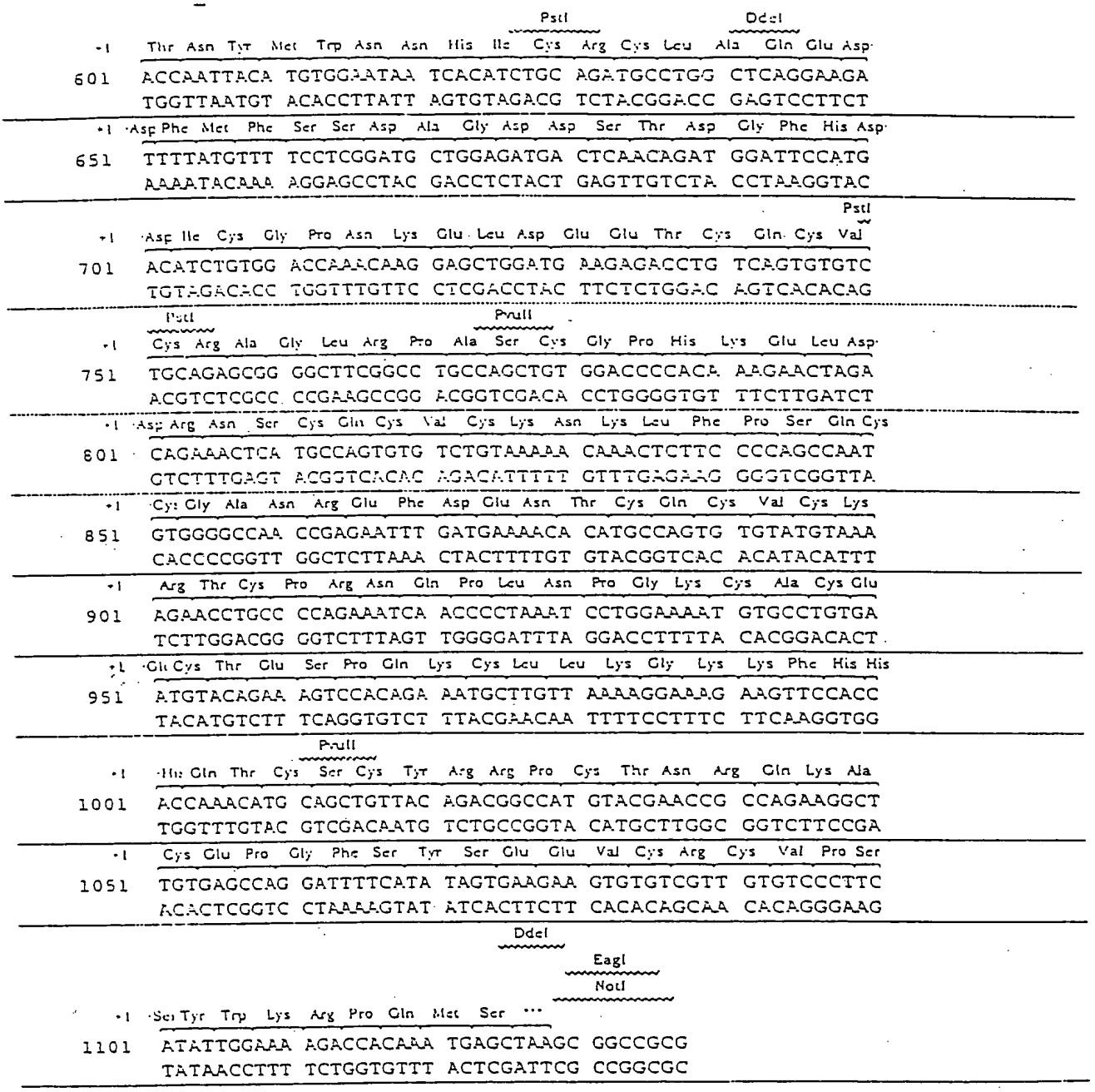
June 4, 1997
Date

Carl-Henrik Heldin

CURRICULUM ITAE 97-05-28

Name:		Carl-Henrik Heldin
Present appointment:		Director Ludwig Institute for Cancer Research (Uppsala Branch) Biomedical Center, Box 595 S-751 24 Uppsala, Sweden Phone: +46-18-160401 Fax: +46-18-160420 E-mail: C-H.Heldin@LICR.uu.se
Home address:		Hesselmans väg 35 S-752 63 Uppsala, Sweden Phone: +46-18-463213
Date and place of birth:		August 9, 1952, Växjö, Sweden
Nationality:		Swedish
Marital status:		Married, two children, born 1982 and 1988
University education:	1971-1975	First four years of Medical School completed (University of Uppsala)
	1972-1981	Bachelor of Science (Mathematics 1 1/2 year, Numeric analysis 1/2 year, Psychology 1/2 year, Greek 1/2 year) completed July 28, 1981 (University of Uppsala)
	1975-1980	Thesis work at Department of Medical and Physiological Chemistry (University of Uppsala). Dissertation May 10, 1980. "Studies on growth factors for human cultured cells".
Academic positions:	1972-1974	Part time teaching positions at Depts of Anatomy, Medical and Physiological Chemistry, and Physiology (in total 200 hours)
	75.07.01- 80.03.31.	Graduate student scholarship at Dept of Medical and Physiological Chemistry combined with a part time teaching position (in total 1100 hours)
	80.04.01- 80.10.31	Research Assistant at Dept of Medical and Physiological Chemistry
	81.01.01- 81.03.31	Lecturer at Dept of Medical and Physiological Chemistry
	81.07.01- 83.12.31	Cancer Research Scholarship from the Swedish Cancer Society
	84.01.01- 85.12.31	Senior Scientist of the Swedish Cancer Society
	86.01.01--	Director, Ludwig Institute for Cancer Research (Uppsala Branch)
	92.08.01--	Professor in Molecular Cell Biology at the Medical Faculty of Uppsala University

Exhibit F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DdeI
EagI
NotI
+1 Ser Tyr Trp Lys Arg Pro Gln Met Ser ***
1101 ATATTGGAAA AGACCACAAA TGAGCTAAGC GGCCGCG
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VEGF-2 419aa Sequence:

```
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+1
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                                 Met His Leu Leu Gly Phe Phe Ser Val Ala

                                        SmaI
                                        XmaI
                                        AvaI        NruI
+1  Ala Cys Ser Leu Leu Ala Ala Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala
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+1  Ala Ala Ala Ala Ala Phe Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro
101 CCGCCGCCGC CGCCTTCGAG TCCGGACTCG ACCTCTCGGA CGCGGAGCCC
    GGCGGCGGCG GCGGAAGCTC AGGCCTGAGC TGGAGAGCCT GCGCCTCGGG

                                                DpnI
                                                BglII
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    CTGCGCCCGC TCCGGTGCCG AATACGTTCG TTTCTAGACC TCCTCGTCAA

                                                                SspI
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    TGCCAGACAC AGGTCACATC TACTTGAGTA CTGACATGAG ATGGGTCTTA
            SspI                    DdeI
+1  Tyr Trp Lys Met Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn
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                                                            PstI
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            PstI                DpnI
                                BglII
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    ACGTCGTGTA ATATTATGTC TCTAGAACTT TTCATAACTA TTACTCACCT

                        SphI
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                NruI                DraI                        AccI
+1  Phe Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr
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    AAACCTCAGC GCTGTTTGTG GAAGAAATTT GGAGGTACAC ACAGGCAGAT

    AccI                                        PstI
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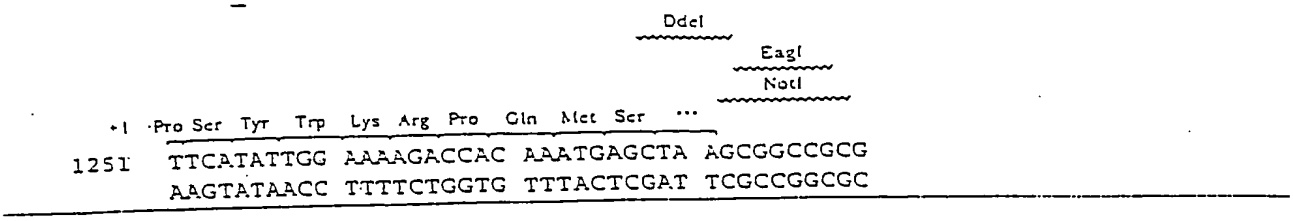
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STRAIN SHEET

STRAIN CMV I STORAGE NUMBER 11:64

PEDIGREE:

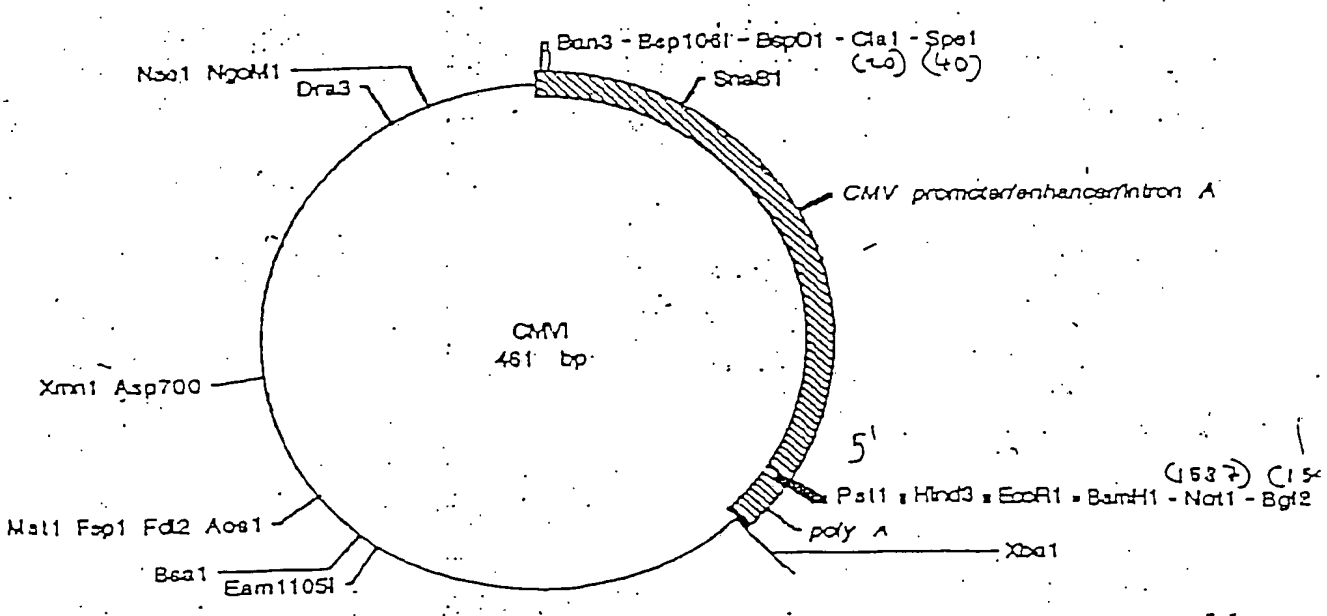
-CMVI was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMVI is 4613 bp.

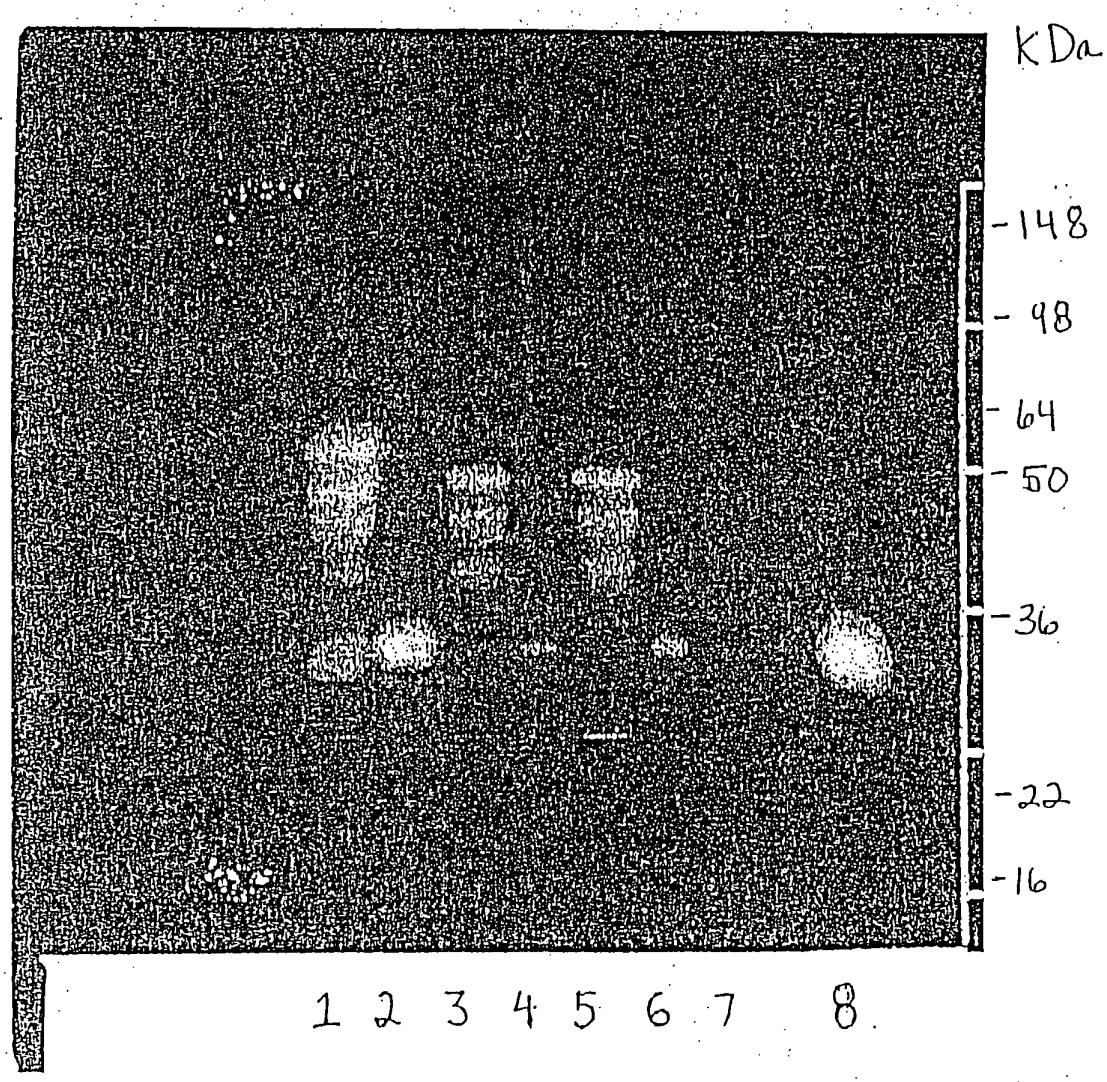
-CMVI uses ampicillin resistance.

-CMV promoter/enhancer + Intron A: nt #'s 1-1566.
-Polylinker: nt #'s 1566-1597 (5'- Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').
-SV40 polyA addition sequence: nt #'s 1598-1745.
-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

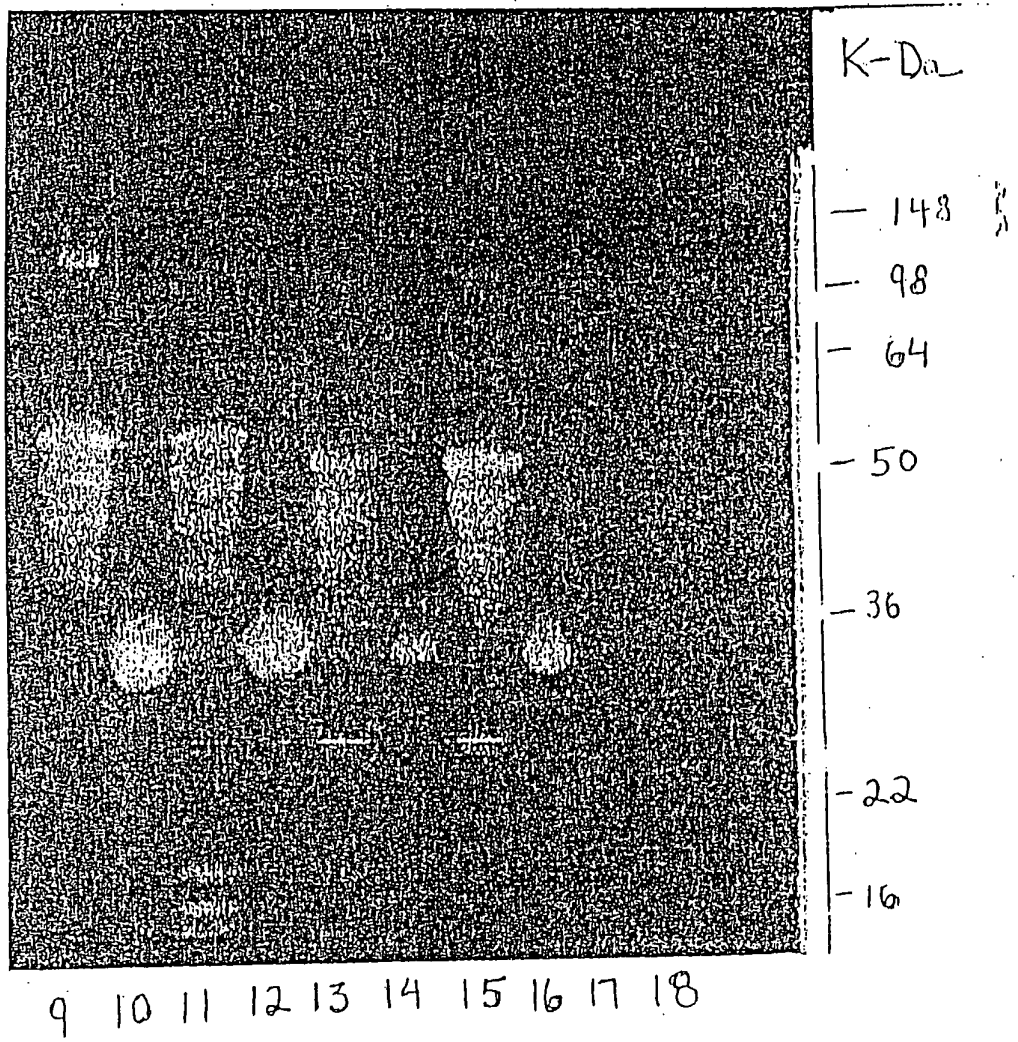
GROWTH REQUIREMENTS:



Appendix 11



Power Declaration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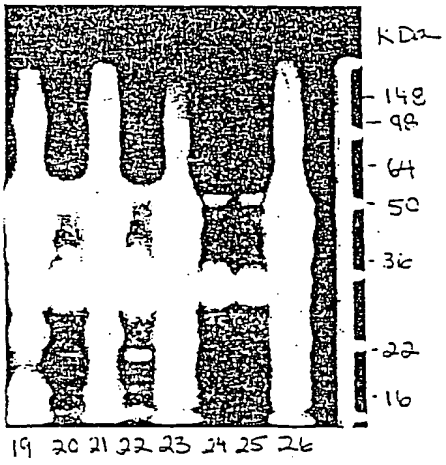
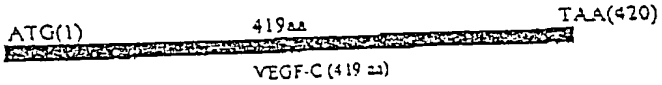


Power Declaration
Figure 1 -- Gel 3

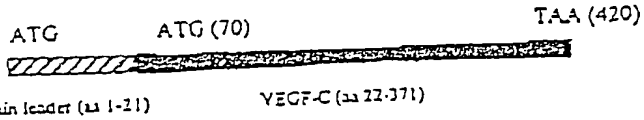
3. To determine whether the 350 amino acid form of VEGF-2 could be secreted from cells when attached to a heterologous signal sequence, I transfected eukaryotic cells with expression vectors encoding the 419 amino acid form of VEGF-2, or the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence. I grew the transfected cells under conditions to allow the cells to express the gene products encoded by the vectors. At various time points, I collected both cell lysates and culture medium and assayed for the presence of VEGF-2, in order to determine if the cells were secreting VEGF-2. The presence of VEGF-2 in either the cell lysates or culture medium was determined by Western Blot analysis of the samples using a polyclonal antibody to VEGF-2, which recognizes both the precursor form and the processed form of VEGF-2

4. The design of the expression vectors used in the study is as follows:

419 amino acid form of VEGF-2 (followed by a STOP codon at position 420):



350amino acid form of VEGF-2 linked to a heterologous signal sequence (followed by a STOP codon at position 420):



5. The nucleotide sequences encoding the 419 and the 350 amino acid forms of VEGF-2 were obtained directly from the American Type Culture Collection (ATCC). ATCC Deposit No. 97149 contains the nucleotide sequence encoding the 419 amino acid form of VEGF-2. ATCC Deposit No. 75698 contains the nucleotide sequence encoding the 350 amino acid form of VEGF-2. The nucleotide sequence encoding the 419 amino acid form of VEGF-2

was engineered to be flanked by an Eco RI site at the 5'end and a Not I site at its 3'end. The second construct contained the nucleotide sequence encoding the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, Ig k-chain leader signal sequence, a recognized signal sequence which was available as of March, 1994, and was engineered to contain a Bam HI site at its 5'end and a Not I site at its 3' end. The sequence of each of these constructs was confirmed to be correct and is detailed in Appendix I, attached hereto.

6. Each VEGF-2 construct was subcloned into the expression vector pCMV-I (see attached plasmid map, attached hereto as Appendix II), so that the VEGF-2 sequences were under the control of a CMV-I promoter, a promoter routinely used as of March, 1994. The 419 amino acid form of VEGF-2 was subcloned into the Eco RI/Not I sites of pCMV-I, while the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, was subcloned into the Bam HI/Not I sites of pCMV-I (see plasmid map, Appendix II).

7. The two VEGF-2 constructs were transiently transfected in duplicate, using the lipofectin method, comparable methods were routinely used as of March, 1994, into the Human Embryonic Kidney cell line, HEK-293 tsA-0, a cell line which was also routinely used as of March, 1994. As a control for transfection efficiency, each construct was co-transfected with the vector pCMV-β-gal. The efficiency of transfection was determined by β-gal staining 48 hours after transfection. As a negative control the vector pCMV-I without an insert was transfected in parallel..

8. The transfection design is as follows:
10 dishes transfected with: pCMV-I-VEGF-419;
10 dishes transfected with: pCMV-I-signal sequence-VEGF-350;
10 dishes transfected: pCMV-I ;
2 dishes transfected with: pCMV-I-VEGF-419 + pCMV-β-gal; and
2 dishes transfected with: pCMV-I-signal sequence-VEGF-350 + pCMV-β-gal.

9. After transfection, DMEM medium containing 3% serum was added to the cells. Aliquots of cell extracts and conditioned medium were prepared from each transfection at: T_0 hours, T_{16} hours, T_{24} hours, T_{48} hours and T_{72} hours, in duplicate.

10. At the time of harvesting the cells and medium were treated as follows:
Medium: Harvested medium was concentrated 3 fold using Centricon 10 concentrator devices. One volume of 2 x PAGE loading dye was added to each sample.
Cell Extracts: The cells were harvested by trypsinization and collected by centrifugation. The cell pellet was resuspended and lysed in 250µl of 1x PAGE loading buffer.

11. To determine the transfection efficiency, dishes transfected with the pCMV-β-gal construct were fixed and stained for β-gal activity 48 hours after transfection. All dishes were found to have the same percentage of transfected cells (70%).

12. Each protein sample was subjected to Western analysis as outlined below. Prior to loading on to a 12% (w/v) Tris-Glycine SDS-Polyacrylamide gel, the samples were boiled for 5 minutes and cooled on ice. The two end lanes of each gel contained the appropriately sized molecular weight markers to estimate the migration rate of proteins predicted to run in the 16 to 85 kDa size range. The samples were electrophoresed according to standard conditions.

13. Following electrophoresis, the samples were transferred to a PVDF membrane. Each membrane was blocked by a one hour incubation in phosphate buffered saline (PBS) containing 3% Bovine Serum Albumin (BSA). The blot was then incubated at 4°C in PBS containing 0.1% BSA and 500ng/ul of purified rabbit anti-VEGF-2 antibody, a polyclonal antibody which recognizes the precursor form and the processed form of VEGF-2. After three 5 minute washes in PBS containing 0.1% Tween, the blot was incubated for 1 hour in PBS containing 0.1% BSA and a 1:3000 dilution of Goat Anti-Rabbit IgG

Horse Radish Peroxidase (HRP) conjugated antibody. The blot was washed three times for 5 minutes in PBS containing 0.1% BSA. The blot was developed with 2ml/blot of ECL detection reagent (obtained from Amersham) for one minute and then exposed directly to Polaroid films for approximately 5 seconds.

14. The result of the experiment is shown in Figure 1, attached hereto. The samples included in the figure are as follows:

Immunoblot analysis of VEGF derivatives transiently expressed in HEK293T cells

Lane	Pellet/Supernatant	350aa -signal / 419aa	T (h) post-transfection
Gel 1			
1	P	419	24
2	S	419	24
3	P	350-signal	24
4	S	350-signal	24
5	P	350-signal	24
6	S	350-signal	24
7	P	negative control	24
8	S	positive control	48
Gel 2			
9	P	419	48
10	S	419	48
11	P	419	48
12	S	419	48
13	P	350-signal	48
14	S	350-signal	48

15	P	350-signal	48
16	S	350-signal	48
17	P	negative control	
18	S	negative control	
Gel 3			
19	P	419	72
20	S	419	72
21	P	419	72
22	S	419	72
23	P	350-signal	72
24	S	350-signal	72
25	S	350-signal	72
26	P	350-signal	72

15. The Western Blot analysis indicates that a doublet of approximately 30kDa was present in the medium collected from the transfection of both the 419 amino acid form of VEGF-2 and the 350 amino acid-VEGF-2 signal sequence constructs (see Figure 1). The secreted protein was visible beginning at 16 hours after transfection. The secreted product from cells containing the 419 amino acid construct and the 350 amino acid-VEGF2 signal sequence construct are approximately the same size.

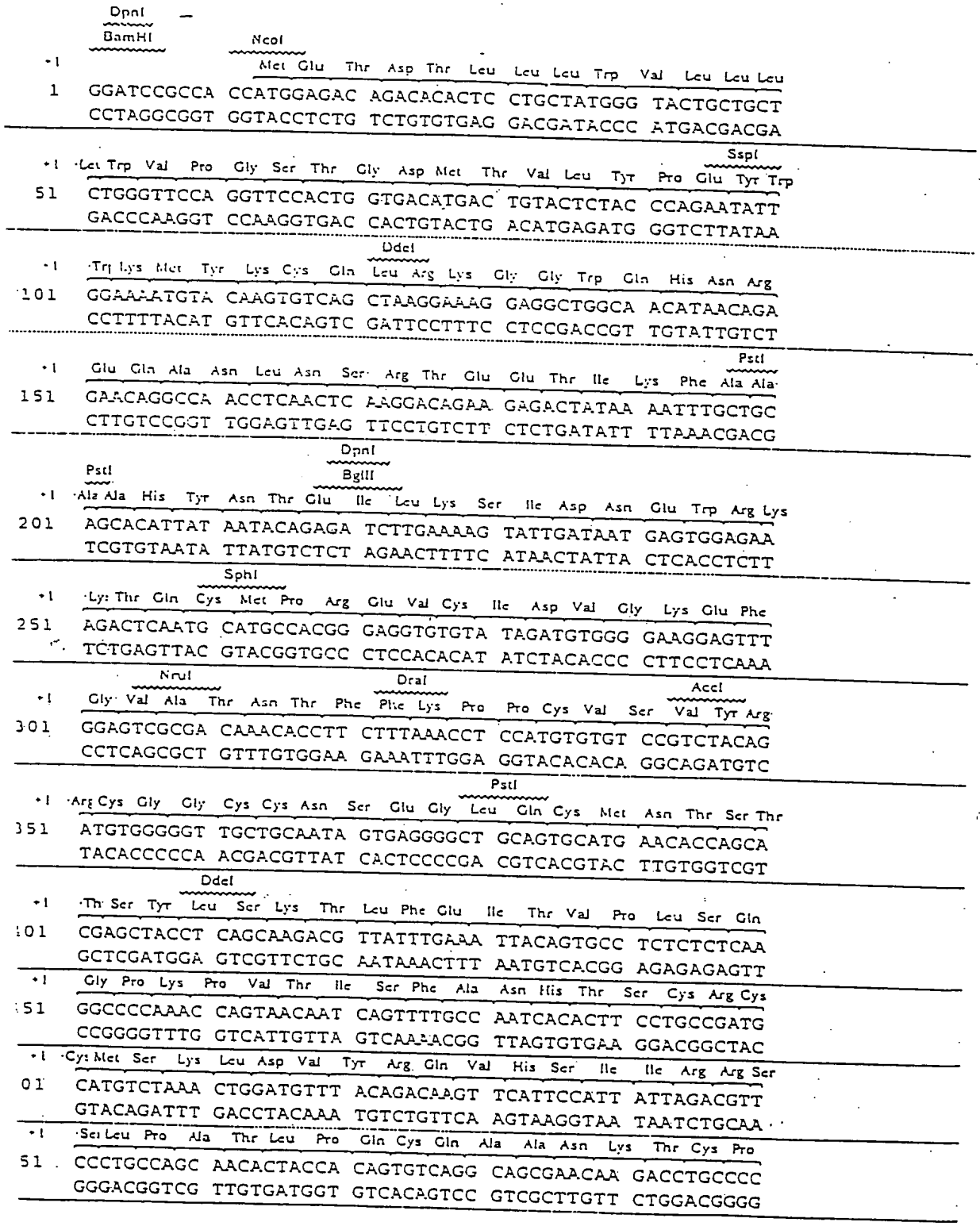
AND I declare that all the statements made in this Declaration are of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Sworn by the said Susan Power, Susan Power at Phillipsburg, New Jersey, on this 5th day of December 2000; before me Maryann White.

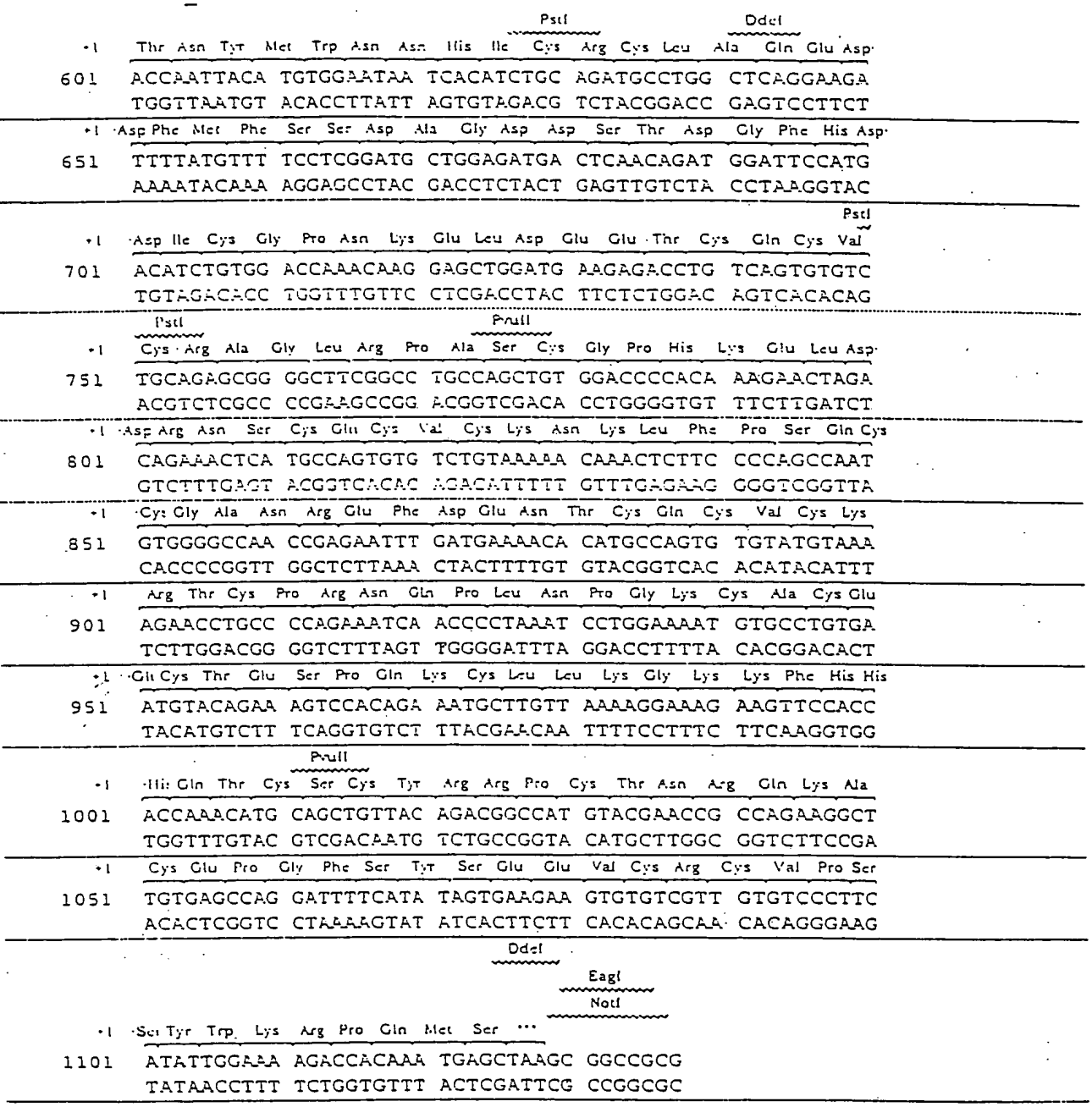
Notary Public

MARYANN WHITE
NOTARY PUBLIC, State of New York
No. 4883761
Qualified in Nassau County
Certification Filed in New York County
Commission Expires January 26, 2001

VEGF-2 350aa +Signal Sequence:



Appendix I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EagI
NotI
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VEGF-2 419aa Sequence:

```
                                   EcoRI
+1                                       Met His Leu Leu Gly Phe Phe Ser Val Ala
1    GAATTCGTGG GTCCTTCCAC CATGCACTTG CTGGGCTTCT TCTCTGTGGC
     CTTAAGCACC CAGGAAGGTG GTACGTGAAC GACCCGAAGA AGAGACACCG

                                   SmaI
                                   XmaI
                                   AvaI      NruI
+1   Ala Cys Ser Leu Leu Ala Ala Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala
51   GTGTTCTCTG CTCGCCGCTG CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG
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+1   Ala Ala Ala Ala Ala Phe Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro
101  CCGCCGCCGC CGCCTTCGAG TCCGGACTCG ACCTCTCGGA CGCGGAGCCC
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                                   DpnI
                                   BglII
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                                                                 SspI
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     SspI                DdeI
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     AccI                                PstI
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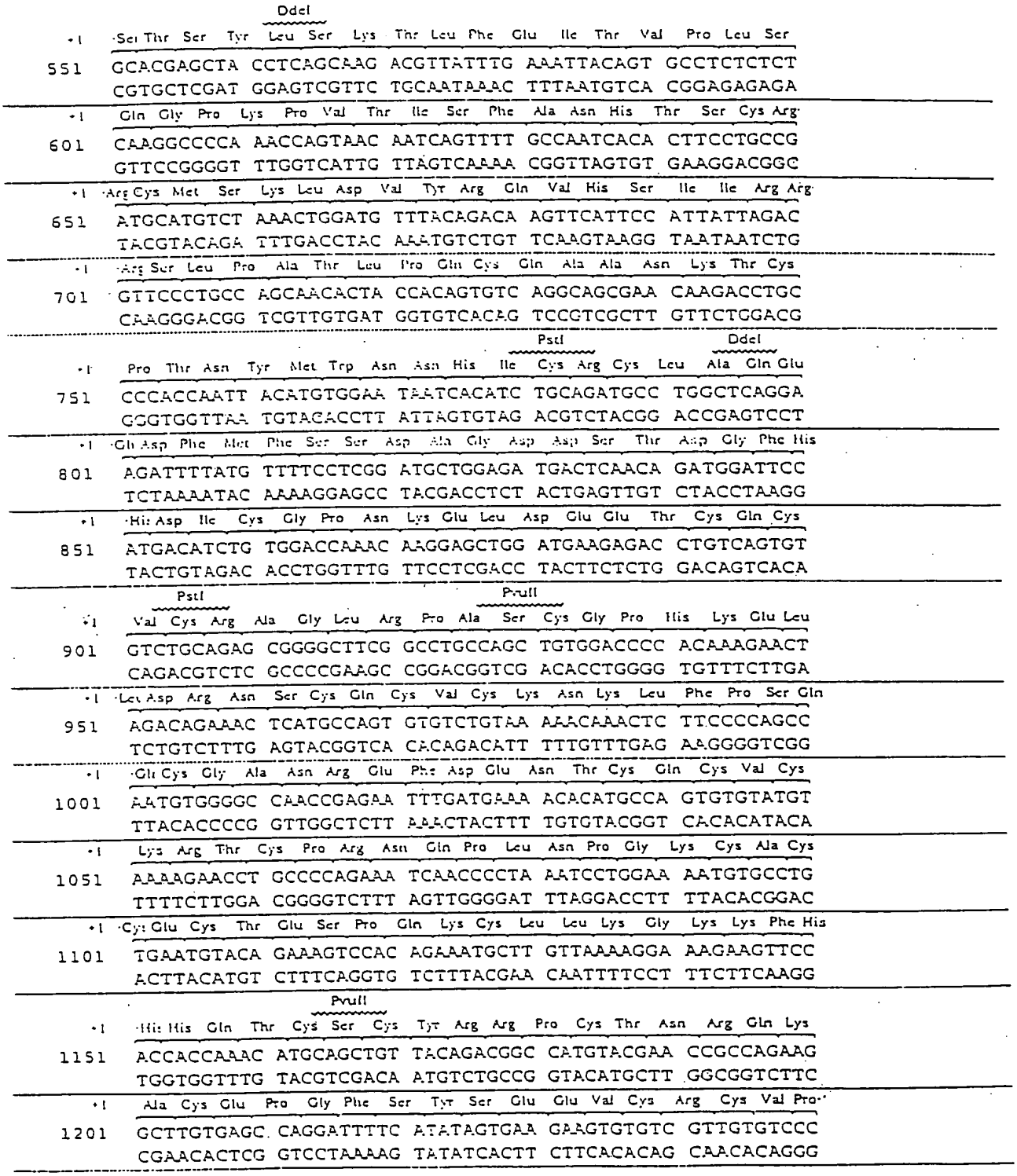
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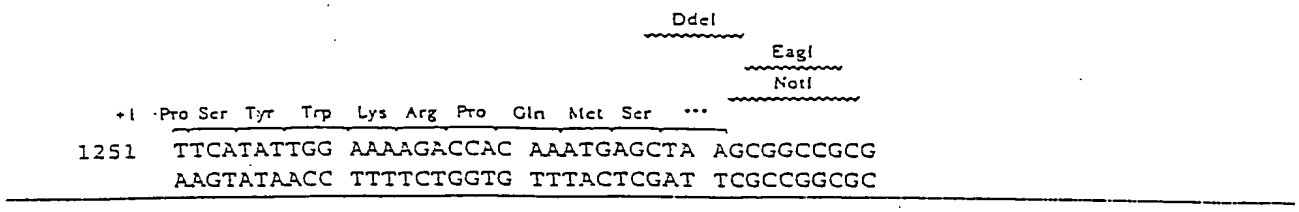
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STRAIN SHEET

STRAIN CMV I STORAGE NUMBER 11:64

PEDIGREE:

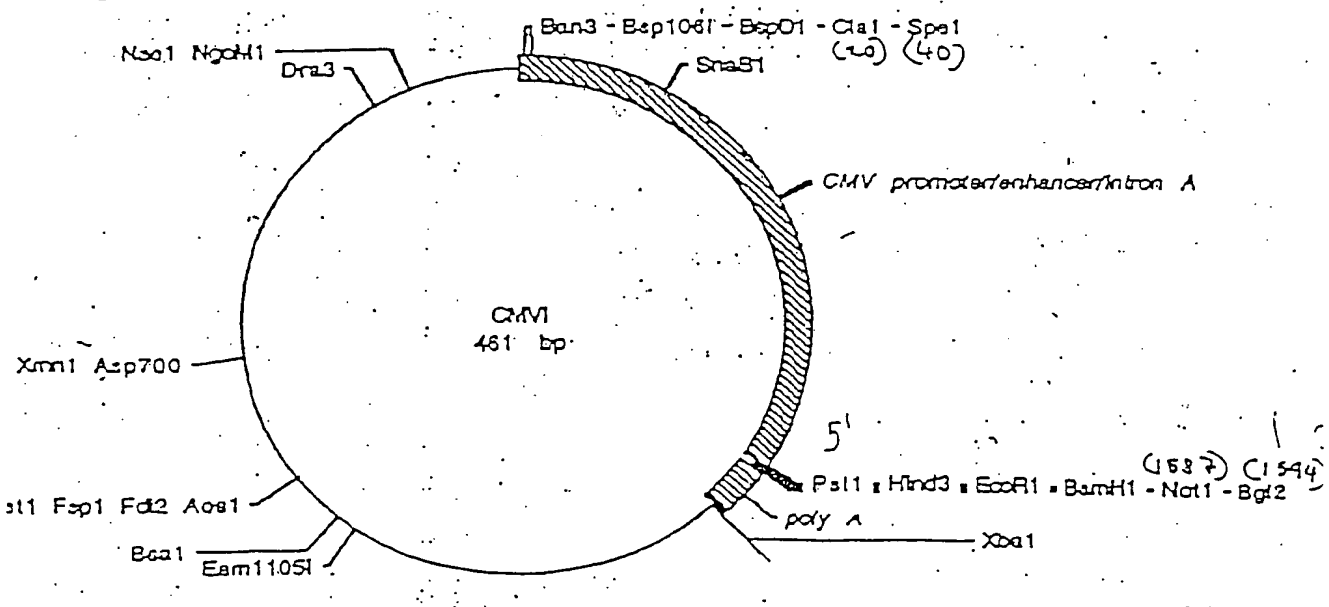
-CMVI was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMVI is 4613 bp.

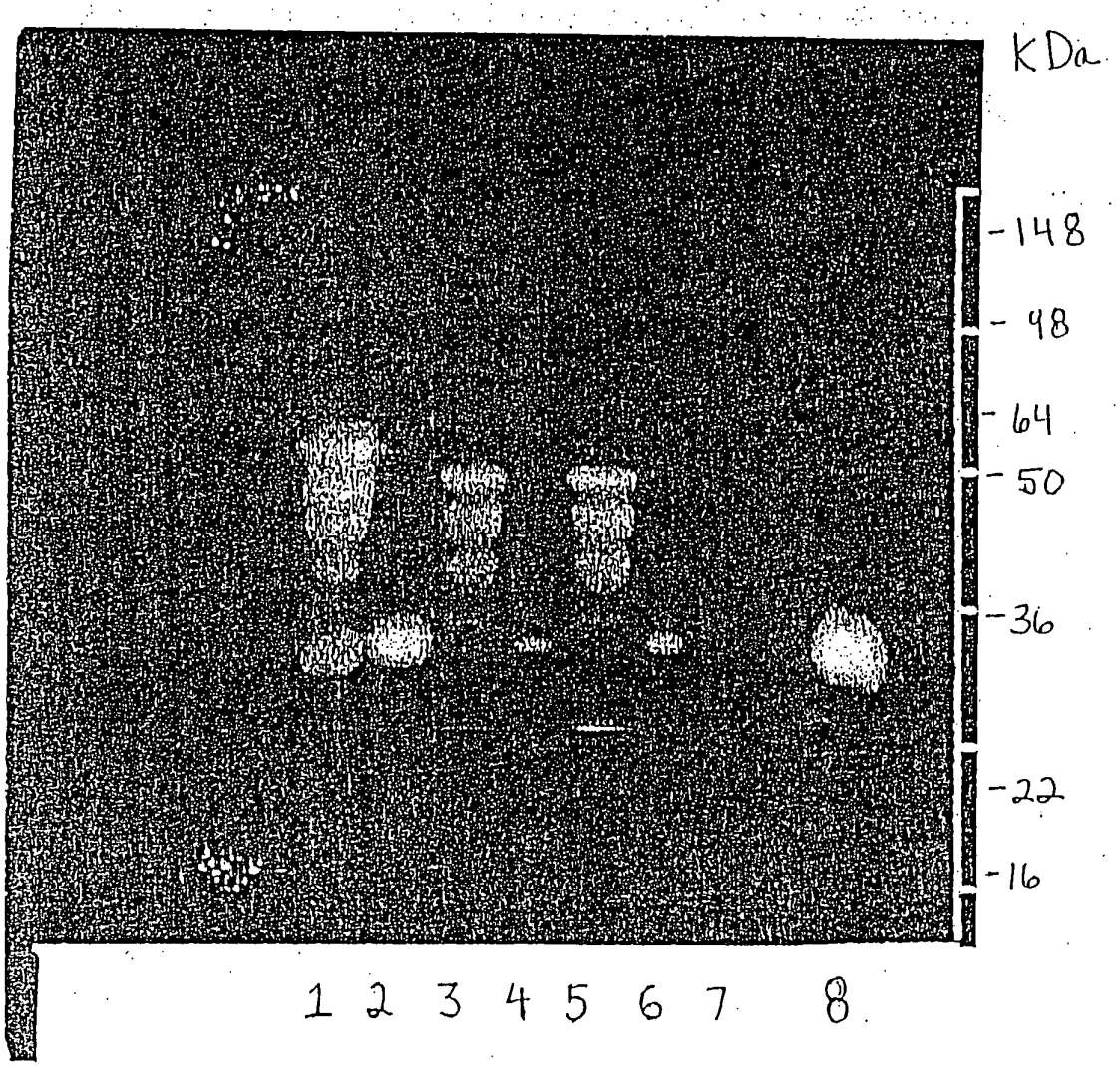
-CMVI uses ampicillin resistance.

-CMV promoter/enhancer + Intron A: nt #'s 1-1566.
-Polylinker: nt #'s 1566-1597 (5'- Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').
-SV40 polyA addition sequence: nt #'s 1598-1745.
-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

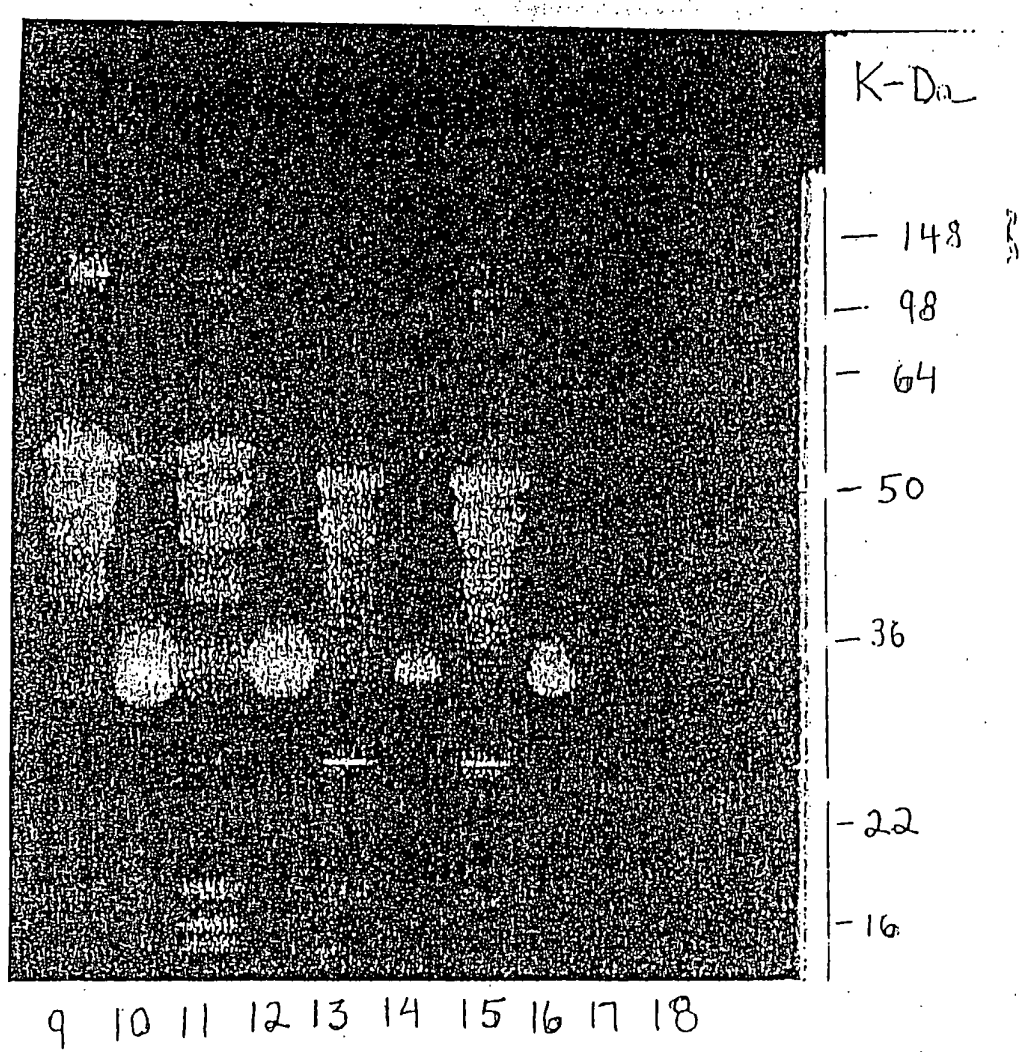
GROWTH REQUIREMENTS:



Appendix II



Power Declaration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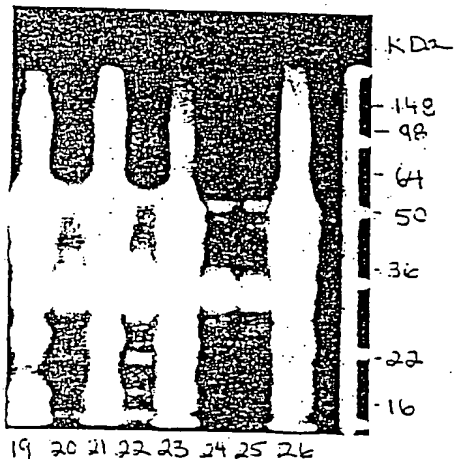

Power Declaration
Figure 1 - Gel 3

Initial Review
BOX AF

sponse Under
37 CFR 1.116- Expedited
Procedure Examining
Group 1814

PATENT
28967/32863

20/c
B.G.J
6/16/97

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Alitalo *et al.*

Serial No: 08/510,133

Filed: August 1, 1995

Title: RECEPTOR LIGAND

Group Art Unit: 1814

Examiner: Lathrop, B.

EXPRESS MAIL LABEL NO:
EM09982708 6US
Date of Deposit: June 11, 1997

I hereby certify that this paper is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

Mark Bonadonna

RECEIVED
JUN 16 1997

AMENDMENT AFTER FINAL ACTION

and

CONDITIONAL PETITION TO REVERSE OR WITHDRAWN ADVERSE PRIORITY DETERMINATION PURSUANT TO 37 C.F.R. §1.181

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In an official action mailed April 11, 1997, the examiner finally rejected claims 1, 8, 9, 13-15, and 19-25 variously under 35 U.S.C. §§ 101 and 112, first paragraph. Claims 2 and 12 were allowed, and claims 16 and 17 were objected to as being dependent upon a rejected base claim, but were otherwise deemed allowable. The applicants respectfully request reconsideration in light of the following amendments and remarks.

AMENDMENTS

In the specification:

At page 24, line 30, after "Figure 9" please insert -- (SEQ ID NOs: 32 and 33) --.

In the claims:

Please cancel claims 3-7 and 11, without prejudice, amend claims 8, 16, 17, 21, 22, and 25, and add new claims 26-28, as shown below.

C'

8. (Three times amended) A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding.

C2

16. (Amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, [according to claim 13,] wherein amino terminal amino acids 2 through 18 of said polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

17. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, [according to claim 1,] said polypeptide being purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

C3

21. (Amended) A polypeptide according to claim [8] 17 further comprising a detectable label.

C3 ord.

22. (Amended) A pharmaceutical composition comprising a polypeptide according to claim [8] 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

C4

25. (Amended) A pharmaceutical composition comprising a polypeptide according to claim [14] 16 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

-- 26. A polypeptide according to claim 8 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.

C5

27. A polypeptide according to claim 8 wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

28. A polypeptide according to claim 16 further comprising a detectable label. --

REMARKS

I. History of claims and explanation of amendments.

The application as filed contained twelve claims. In a preliminary amendment (Paper No. 10) dated August 12, 1996, claims 1-4, 6, 8-9, and 12 were amended, and claims 13-19 were added to the application. In an amendment dated February 10, 1997, the applicants canceled claims 10 and 18, amended claims 1, 8, 9, 14, and 17, and added new claims 20-25. In the present amendment, the applicants cancel claims 3-7 and 11, amend claims 8, 16, 17, 21, 22, and 25, and add new claims 26-28. Thus, upon entry of the foregoing amendments, claims 1-2, 8-9, 12-17, and 19-28 would be pending. A copy of the claims in their amended forms is appended hereto.

The nature of each claim amendment is discussed below in the remarks pertaining to each claim.

New claim 26 depends from claim 8 and further limits claim 8 by adopting a suggestion of the examiner with respect to subject matter that the specification enables. Support for the limitation "within amino acids 1-180 of SEQ ID NO: 33" is found in the specification at p. 28, lines 1-3. New claim 27 further limits claim 26 by reciting a specific amino terminal amino acid residue. The particular amino terminus that is recited in claim 27 corresponds to the amino terminus recited in claim 16. This amino terminus finds written support at p. 19, lines 17-19 of the specification.

II. Restriction Requirement

The applicants have canceled non-elected claims 3-7 and 11 without prejudice.

III. **The Applicants respectfully request issuance of an advisory action wherein the Patent Office reverses as incorrect, or withdraws as inappropriate, its determination that no claims in the present application are afforded priority to U.S.S.N. 08/340,011.**

In the outstanding official action, the examiner has asserted, for the first time, that no claims in the present application are entitled to priority based upon U.S.S.N. 08/340,011, filed November 14, 1994, *because of an asserted lack of written description* under 35 U.S.C. §112, first paragraph.[1] For the reasons set forth below, this determination is legally and factually incorrect. Moreover, the right of priority has no bearing on the patentability of any claim at

---

[1] In its first official action, the examiner made an initial determination that no claims were afforded priority by the '011 application, because of an *asserted absence of enabling disclosure*. However, that initial determination was made without any consideration of the preliminary amendment portion of U.S.S.N. 08/340,011 (a significant omission, since the '011 application is a Rule 62 continuation-in-part of an earlier application, and the preliminary amendment portion of the '011 application is highly pertinent to the priority issue). In the outstanding final action, the priority determination based on lack of enablement has properly been withdrawn. However, the examiner has, for the first time, raised a new objection to the priority claim, based upon an asserted lack of written description.

this time, and therefore, is an inappropriate subject for Patent Office determination.

A. The applicants respectfully request entry into the record and consideration of the expert declaration of Dr. Carl-Henrik Heldin filed herewith.

The Patent Office's reviewing court has explicitly approved of the use of declarations which offer factual evidence to help resolve the issue of "written description" in a patent application, and has held that failure to accord appropriate weight to such declarations constitutes legal error. See *In re Alton*, 37 U.S.P.Q. 1578, 1583 (Fed. Cir. 1996). The applicants have filed herewith the expert declaration of Dr. Carl-Henrik Heldin (the "Heldin declaration") to offer a factual explanation as to *why* one of ordinary skill in the art would have understood the 1994 priority application to describe the invention presently being claimed. Since the examiner raised the written description issue for the first time in the outstanding final official action,[2] the applicants respectfully request entry of this declaration into the record and consideration thereof with respect to the issue of written description.

B. The determination that no claims are entitled to priority is legally and factually incorrect.

The law is clear that original claims (i.e., claims contained in the patent application as filed) comply with the written description requirement of §112, because *original claims constitute their own description*. See *In re Koller*, 204 U.S.P.Q. 702, 706 (C.C.P.A. 1980). Moreover, later added claims of similar scope and wording are described by original claims. *Id*.

In the present case, the applicants' 1994 priority application (the '011 application) contained original claims to an Flt4 ligand. For example, original claim 31 recites, "A ligand which specifically binds to an FLT-4 receptor

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[2] The written description issue was not necessitated solely by amendments made by the applicants in response to the first action on the merits, and therefore could have been raised by the Patent Office prior to the issuance of a final action.

tyrosine kinase." By way of comparison, claim 1 of the present application recites, "A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase."

Claim 1 is unquestionably of similar scope and wording to claim 31 as originally filed. Whereas original claim 31 was directed to "a ligand," claim 1 is directed to "a purified and isolated polypeptide." However, the 1994 priority application clearly states that the ligand of the invention is a purified protein. (See, e.g., preliminary amendment to U.S.S.N. 08/340,011 dated November 14, 1994, at p. 15: "The purified biologically active ligand protein . . . .") Whereas original claim 31 was directed to binding to Flt4 receptor tyrosine kinase, claim 1 clarifies that the ligand binds to *the extracellular domain* of Flt4. However, the 1994 priority application clearly states that the ligand protein binds to the Flt4 *extracellular domain*. (See, e.g., preliminary amendment to U.S.S.N. 08/340,011 dated November 14, 1994, at p. 11: "The above experiments prove that the ligand binds to the recombinant FLT4 EC domain.") Finally, original claim 31 recites that the ligand "specifically binds" whereas claim 1 is directed to "high affinity" binding. However, this difference merely adopts preferred claim language suggested by the examiner in the course of prosecution. Thus, claim 1 is unquestionably of similar scope and wording to an original claim of the '011 patent application. (See the Heldin declaration at ¶ 6.) Accordingly, original claims in the '011 patent application provide written description support for claim 1 of the present patent application. See *In re Koller*, 204 U.S.P.Q. at 706.

Claim 19, which depends from claim 1 and recites that the polypeptide further comprises a detectable label, finds written description support in original claim 33 of the 1994 priority application. See *In re Koller*, 204 U.S.P.Q. at 706; see also the Heldin declaration at ¶ 7.

Claim 17 (which depended from claim 1 but has been rewritten as an independent claim incorporating the limitations of claim 1) is similar to claim 1 and additionally recites that the polypeptide is purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, the cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity

purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase. These additional limitations find explicit written description support in the 1994 priority application in Examples 12 and 15: Example 12 teaches that conditioned media from the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) produces a soluble ligand for Flt4 that binds to recombinant Flt4 extracellular domain and that can be purified using the Flt4 EC domain in affinity chromatography; Example 15 describes such affinity chromatography. (See the preliminary amendment to the '011 application at pp. 8-11 and 15.) Thus, claim 17 finds written description support in the original claims of the 1994 priority application coupled with the written description provided in Examples 12 and 15. (See the Heldin declaration at ¶ 8.)

Claim 21 as amended is identical to claim 19 except that claim 21 depends from claim 17. Thus, claim 21 finds written description support in the 1994 priority application for the reasons outlined above with respect to claims 17 and 19.

Claim 14 recites "A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase." Written description support for "a purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase" is discussed above in relation to claim 1. Example 12 in the 1994 priority application teaches that the Flt4 polypeptide ligand stimulates Flt4 tyrosine phosphorylation in mammalian cells that express Flt4 receptor tyrosine kinase. (See the preliminary amendment to the '011 application at pp. 8-11; see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or fragment thereof, which is capable of stimulating the Flt4 receptor . . . .") Thus, claim 14 finds written description support in the original claims of the '011 application coupled with the written description provided in Example 12. (See the Heldin declaration at ¶ 9.)

Claims 13 depends from claim 1 and recites that the polypeptide has an apparent molecular weight of approximately 23 kD as assessed by SDS-

PAGE under reducing conditions. The approximate 23 kD molecular weight is an *inherent property* of the Flt4 ligand that the '011 application teaches one how to purify from the PC-3 conditioned medium. (See the Heldin declaration at ¶¶ 10 and 11.A.) As such, the inclusion of this property in the present application and in claim 13 does not deprive claim 13 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*, 33 U.S.P.Q.2d 1274, 1276 (Fed. Cir. 1995) ("A claim in a CIP application is entitled to the filing date of the parent application when the claimed invention is described in the parent specification in a manner that satisfies, inter alia, the description requirement of 35 U.S.C. §112. . . . [T]he later explicit description of an inherent property does not deprive the product of the benefit of the filing date of the earlier application."); *Ex parte Yamaguchi*, 6 U.S.P.Q.2d 1805, 1807 (PTO Bd. App. 1987) (Claim to compound characterized by a particular x-ray diffraction spectrum has written description support in earlier application that teaches the compound, notwithstanding the absence of any teaching of the x-ray diffraction pattern in the earlier application, because a compound and all of its properties are inseparable); see also *Application of Davies*, 177 U.S.P.Q. 381, 385, 475 F.2d 667, 671-72 (C.C.P.A. 1973) ("[W]e see no impediment to the present applicants' refiling their application and incorporating a discussion of the allegedly unobvious properties [of the invention] while retaining the effective date of the application involved here through §120.")

Claim 15 depends from claim 14 and further recites that the polypeptide "comprises an amino acid sequence set forth in SEQ ID NO: 13." This partial amino acid sequence is an *inherent property* of an Flt4 ligand that the 1994 priority application teaches one how to purify from the PC-3 conditioned medium. (See the present application at p. 19, lines 9-19 (teaching that Flt4 ligand affinity purified from PC-3 medium has an amino terminal amino acid sequence set forth in SEQ ID NO: 13); see also the Heldin declaration at ¶¶ 10 and 11.B.) As such, the inclusion of this property in the present application and in claim 15 does not deprive claim 15 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies*, *supra*.

Claim 16 (which depended from claim 13 but has been rewritten in independent form) additionally recites amino terminal amino acid sequence information of the claimed polypeptide. The recited amino acid sequence is an *inherent property* of the Flt4 ligand that the 1994 priority application teaches one how to purify from PC-3 conditioned medium.[3] (See the Heldin declaration at ¶¶ 10 and 11.C.) As such, the inclusion of this property in the present application and in claim 16 does not deprive claim 16 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies, supra*.

New claim 28 is identical to claim 19 except that claim 28 depends from claim 16. Thus, claim 28 finds written description support in the 1994 priority application for the reasons outlined above with respect to claims 16 and 19.

Claim 23 depends from claim 14 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." This molecular weight limitation is an *inherent property* of an Flt4 ligand that the 1994 priority application teaches one how to purify from the PC-3 conditioned medium, as discussed above with respect to claim 13. (See also the Heldin declaration at ¶¶ 10 and 11.D.) As such, the inclusion of this property in the present application and in claim 23 does not deprive claim 23 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies, supra*.

Moreover, the foregoing is not intended to be a complete list of those claims which find written description support in the specification. See the Heldin declaration at ¶11.)

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[3] Moreover, the 1994 priority application teaches to determine the amino terminal amino acid sequence. (See preliminary amendment to '011 application at p. 15 (Example 15).)

C. The Patent Office's reliance on the *Fiers* case is improper.

In dismissing the applicants' priority claim on written description grounds, the Patent Office relied upon the Federal Circuit's decision in *Fiers v. Revel*, 25 U.S.P.Q.2d 1601, 1606 (Fed. Cir. 1993). (Official action at pp. 2 and 3.) However, the *Fiers* opinion was rendered on its own distinct set of facts, and was rendered in the context of the state of the art in 1979-81 (i.e., about 13-15 years prior to the applicants' 1994 filing date). Since the issue of written description is factual in nature, *In re Alton*, *supra*, 37 U.S.P.Q.2d at 1580, the examiner's reliance upon a legal opinion rendered on different facts, and in a much earlier period of the art of molecular biology, is highly suspect from the outset.

1. The present application is distinguishable from the facts of the *Fiers* case because the present application teaches a method of preparing the claimed protein as a natural isolate.

The *Fiers* opinion was based on the premise that a written description of a DNA invention requires the same degree of specificity as a conception of a DNA invention. *Fiers*, 25 U.S.P.Q.2d at 1606. Citing its earlier opinion in *Amgen v. Chugai Pharmaceutical Co.*, 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991), the Court acknowledged that conception of a DNA can occur where one is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. *Fiers*, 25 U.S.P.Q.2d at 1604. In the present case, the 1994 priority application is able to define the Flt4 ligand protein by a method of preparation (e.g., affinity purification using the Flt4 extracellular domain) and by chemical characteristics (e.g., a polypeptide that is capable of stimulating the Flt4 receptor and regulating vascular endothelial cells). Thus, under the standards articulated in the *Fiers* and *Amgen* cases for DNA inventions, the 1994 priority application contains a written description of the Flt4 ligand protein invention claimed herein.

2. The present application is distinguishable from *Fiers* because the invention presently claimed pertains to a purified protein.

In *Fiers*, the Federal Circuit rendered an opinion as to that which is required under §112, first paragraph, for an adequate written description of a DNA invention. The invention claimed in the present application is not a DNA invention;[4] the invention pertains to a purified protein, and the issue concerns whether a priority application contains a sufficient written description of that protein invention. The examiner has failed to articulate why a factual determination in *Fiers* pertaining to a DNA invention is relevant to a factual determination pertaining to a protein invention in the present case.[5] Accordingly, the examiner has failed to meet his burden of establishing a *prima facie* case of lack of written description.

Since the *Fiers* holding is distinguishable on its facts and also was rendered in the context of the state of the art in 1979-81, i.e., about 13-15 years prior to the applicants' 1994 filing date, the *Fiers* opinion fails to support the examiner's written description objection.

D. The right of priority has no bearing on the patentability of any claim at this time, and therefore, is an inappropriate subject for Patent Office determination.

The Manual of Patent Examining Procedure instructs that a priority determination should be made during *ex parte* prosecution *only* when an intervening reference is found, upon which a rejection under §102 or §103 would be made:

> The only times during *ex parte* prosecution that the examiner considers the merits of an applicant's claim of

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[4] The Patent Office has deemed the DNAs taught in the application to constitute a distinct invention.

[5] In this regard, the Patent Office's attention is directed to *Scripps Clinic v. Genentech Inc.*, 18 U.S.P.Q. 1001 (Fed, Cir. 1991), an opinion issued contemporaneously with the *Amgen* opinion and pertaining to a purified protein invention. The Patent at issue in the *Scripps* case (Reissue Patent No. 32,011) contained claims to a purified protein (Factor VIII:C) and to an affinity method of purifying the protein. No amino acid sequence description was required under §112, first paragraph, for the Patent Office to issue or to reissue this patent.

> priority is when a reference is found with an effective date between the date of the foreign filing and the date of filing in the United States and when an interference situation is under consideration. If at the time of making an action the examiner has found such an intervening reference, he or she simply rejects whatever claims may be considered unpatentable thereover, without paying any attention to the priority date . . . .

(M.P.E.P.((6th Ed., Rev. 2) §201.15.)

The outstanding final action constitutes the first time that the Patent Office has raised its written description objection as a basis for refusing to afford priority to the '011 application.[6] However, there are no prior art rejections based upon intervening references in the outstanding action. Accordingly, under the Patent Office's own procedures, it was inappropriate to consider the merits of the priority claim in the official action.

E. Conditional Petition to Reverse or Withdrawn Adverse Priority Determination.

Should the examiner refuse to reverse or withdraw the adverse priority determination that was made for the first time in the final official action, the applicants hereby petition the commissioner to reverse this determination as improper, or, in the alternative, to withdraw this determination as premature and expunge from the file all mention of this premature determination. The facts in support of reversal of the priority determination are provided in parts A-C, above, and in the Declaration of Dr. Heldin filed herewith. The facts in support of withdrawal of the premature determination are provided in part D, above. In the event of withdrawal, the applicants respectfully submit that all mention of the priority determination in the final official action and this submission by the applicants should be expunged from the file, so as not to taint the file history of the eventual patent in a manner adverse to the applicants.

The priority issue is properly the subject of a petition because the priority determination is not pertinent to any rejection and, therefore, is not

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[6] See note 1, *supra*.

subject to review by the Board of Patent Appeals and Interferences. See M.P.E.P. §706.01.

The applicants hereby authorize the commissioner to charge any necessary petition fee associated with this conditional petition to Deposit Account No. 13-2855. This petition has been timely filed within two months of the mailing of the final official action that contains the adverse priority determination at issue.

IV. **The amendments to claim 8 render moot the rejection of claims 8-9 and 19-20.**

In paragraph 9 of the outstanding official action, the examiner rejected claims 8-9 and 20-22 under 35 U.S.C. § 101, asserting that these claims read on a product of nature, because claim 8 fails to recite a "purified and isolated" polypeptide. (Office action at p. 5.)

In response, the applicants have amended claim 8 to recite, "A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding." Thus, amended claim 8 does not read on a product of nature, rendering the rejection of claim 8 (and claims 9 and 19-20 which depend therefrom) moot. Since this amendment adopts a suggestion of the Patent Office and removes an issue for appeal, entry of the amendment and withdrawal of the rejection is respectfully requested.

V. **The amendments to claims 16 and 17 place these claims in condition for allowance.**

In paragraph 15 of the outstanding action, the Patent Office objected to claims 16 and 17 as being dependent upon a rejected base claim, but indicated that these claims would be allowable if rewritten in independent form. (Office action at p. 11.) In response, the applicants have rewritten claims 16 and 17 in independent form, incorporating all of the limitation of the base claim and any intervening claims. Accordingly, claim 16 and 17 are now in condition for allowance.

VI. The amendments to claims 21, 22, and 25 place these claims in condition for allowance; and new claim 28 is in condition for allowance.

Claims 21, 22, and 25 have been amended to depend from and further limit claims 16 and 17. New claim 28 is identical to claim 21 and depends from claim 16. Because the subject matter of claims 16 and 17 has been deemed allowable, the amendment of claims 21, 22, and 25 (and addition of claim 28) to depend from claims 16 and 17 also places these claims in condition for allowance. Accordingly, entry of these amendments and allowance of claims 21, 22, 25, and 28 is respectfully requested.

VII. The Patent Office's rejections of clams 1, 8, 9, 13-15, and 19-25 under §112, first paragraph, for lack of enablement improperly ignore both guidance provided in the specification and the skill of those in the art.

In paragraphs 10-13 of the official action, the examiner articulated his basis for maintaining rejections of claims 1, 8, 9, 13-15, and 19-25 under §112, first paragraph, for lack of enablement. The Patent Office admits that fragments of the protein of SEQ ID NO: 33 can be made, but asserts that undue experimentation would be required to screen all fragments of SEQ ID NO: 33 to determine which fragments bind the receptor:

> The examiner argues that the claim limitation of binding the receptor must be met by testing all fragments encompassed by the claims, which in this case are not limited in any way.

(official action at p. 7.)[7]

The Patent Office's insistence that it is necessary to test all fragments of SEQ ID NO: 33 ignores the scientific ability of one of ordinary skill in the art. Importantly, one of ordinary skill in the art would not conduct experimentation by haphazardly making <u>all</u> of the possible fragments of SEQ ID NO: 33 and testing their ability to bind the receptor. An artisan of ordinary skill understands that each fragment that is screened provides guidance as to that

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[7] Claim 8 encompasses only polypeptides which are capable of binding the Flt4 receptor. To the extent that the examiner has interpreted claim 8 (or similarly limited claims) to "encompass" all fragments of SEQ ID NO: 33, the examiner has ignored a limitation of claim 8 and thereby erroneously construed the claim.

portion of SEQ ID NO: 33 that is effective for binding, and that portion which is not.[8] An artisan of ordinary skill also understands techniques for accelerating a screening process,[9] and techniques for screening multiple polypeptides *simultaneously*. Thus, the examiner's reasoning greatly overstates both the quantity and the nature of the experimentation required to practice the invention as claimed.

In this regard, the application provides explicit guidance for screening fragments of SEQ ID NO: 33 to determine a portion effective to permit Flt4 binding. Although SEQ ID NO: 33 contains 350 amino acids, the specification provides guidance that the region critical for receptor activation is contained within its first approximately 180 amino acid residues.

> By extrapolation from studies of the structure of the related platelet derived growth factor (PDGF, reference Heldin, et al., Growth Factors 8, 245-252, 1993) one determines that the region critical for receptor activation by the Flt4 ligand is contained within its first approximately 180 amino acid residues. (Specification, pp. 27-28.)

To determine which fragments contain a sufficient portion of SEQ ID NO: 33 to permit binding, the specification also outlines a specific protocol. The specification teaches one skilled in the art to (a) generate progressive deletion products of the Flt4 ligand cDNA; (b) express these modified cDNAs; and (c) assay the resulting truncated protein forms, e.g., by studying their ability to induce Flt4 autophosphorylation. (Specification at, e.g., p. 27, lines 23-29.) These teachings serve to both provide guidance for predicting the portions of

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[8] For example, a determination that a polypeptide comprising residues 34-180 of SEQ ID NO: 33 is effective to permit binding to Flt4 and that a polypeptide comprising residues 181-350 is ineffective to permit binding would provide significant guidance as to that portion of SEQ ID NO: 33 to further screen for effective fragments. Thus, the assertion that it would be necessary to screen "all" fragments of SEQ ID NO: 33 to practice the claimed invention relies upon the false assumption that individual screening assays will be performed without knowledge gained from prior screenings.

[9] For example, it is within the skill of the art to synthesize spaced deletion mutants (e.g., residues 34-350, 34-330, 34-310, etc.) from SEQ ID NO: 33, rather than successive deletion mutants (34-350, 34-349, 34-348 . . .), to more rapidly identify effective portions for binding Flt4.

SEQ ID NO: 33 that are effective to permit Flt4 binding; and (2) reduce the amount of experimentation required to determine the minimum portion of SEQ ID NO: 33 that is critical for receptor binding.

Moreover, as explained above, it is within the skill of the art to synthesize deletion mutants of SEQ ID NO: 33 that have been spaced intermittently (e.g., residues 34-180, 34-160, 34-140, 34-120, etc.), rather than synthesize every possible successive deletion mutant (34-180, 34-179, 34-178, 34-177 . . .), to more rapidly identify effective portions for binding Flt4. Furthermore, the skilled artisan is capable of synthesizing and screening several such deletion fragments simultaneously, in parallel experiments. Thus, the examiner's assertions that it is necessary to screen every fragment of SEQ ID NO: 33, that the specification lacks guidance, and that the amount of screening required constitutes undue experimentation is improper. See *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988) ("Enablement is not precluded by the necessity for some experimentation such as routine screening. . . . The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.")

VIII. Summary

For the foregoing reasons, the applicants respectfully request reconsideration, withdrawal of all claim rejections and objections to the specification, withdrawal of the notation that no claims are afforded priority to the parent application, and allowance of claims 1-2, 8-9, 12-17, and 19-28.

Respectfully submitted,

June 11, 1997

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Appendix of claims

1. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase.

2. (Amended) A purified and isolated polypeptide comprising an amino acid sequence shown in SEQ ID NO: 33.

8. (Three times amended) A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding.

9. (Twice amended) A polypeptide according to claim 8 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

12. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 2 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

13. A polypeptide according to claim 1 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

14. (Amended) A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

15. A purified and isolated polypeptide according to claim 14, said polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 13.

16. (Amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, wherein amino terminal amino acids 2 through 18 of said polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

17. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, said polypeptide being purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

19. A polypeptide according to claim 1 further comprising a detectable label.

20. A polypeptide according to claim 8 which is capable of binding the extracellular domain of Flt4 receptor tyrosine kinase with high affinity and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

21. (Amended) A polypeptide according to claim 17 further comprising a detectable label.

22. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

23. A polypeptide according to claim 14 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

24. A polypeptide according to claim 14 comprising a portion of SEQ ID NO: 33 effective to permit binding to Flt4 receptor tyrosine kinase and stimulation of Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

25. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 16 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

26. A polypeptide according to claim 8 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.

27. A polypeptide according to claim 8 wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

28. A polypeptide according to claim 16 further comprising a detectable label.

PATENT
28967/32863

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

Alitalo et al.

Serial No.: 08/510,133

Filed: August 1, 1995

For: RECEPTOR LIGAND

Group Art Unit: 1814

Examiner: Lathrop, B.

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I hereby certify that this paper and the documents referred to as enclosed herewith are being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Mark Bonadonna

Declaration of Carl-Henrik Heldin
Pursuant to 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Carl-Henrik Heldin, hereby state as follows:

1. I am Director and member of the Uppsala Branch of Growth Regulation of the Ludwig Institute of Cancer Research (the Ludwig Institute) in Uppsala, Sweden. My curriculum vitae is attached hereto as Exhibit A.

2. I understand that on 01 August 1995, Dr. Kari Alitalo and Dr. Vladimir Joukov (as inventors) filed U.S. Patent Application Serial No. 08/510,133 (hereinafter "the 1995 application"), directed to a polypeptide ligand for Flt4 receptor tyrosine kinase; fragments thereof; a polynucleotide encoding the ligand; vectors and host cells comprising the polynucleotide; and

antibodies reactive with the ligand. I understand that the Ludwig Institute now has an ownership interest in this application.

3. I further understand that, during examination of the 1995 application by the U.S. Patent and Trademark Office (the Patent Office), the examiner has taken the position that U.S. Patent Application Serial No. 08/340,011, filed on 14 November 1994 ("the 1994 application") does not contain a written description of the polypeptide invention that is being claimed in the 1995 application. I have been asked by the Ludwig Institute to review the 1994 and 1995 applications and to provide a factual analysis of whether the 1994 application contains a written description of the invention that is being claimed in the 1995 application.

4. I understand that the claims in a patent application are the portion of a patent application that defines the invention for which patent applicants seek patent protection. I further understand that patent applications are written for the practitioner of ordinary skill in the pertinent scientific field. In the scientific specialties or subdisciplines which fall within the general category of "cellular and molecular biology," the reader of ordinary skill in 1994 and 1995 (hereinafter "the reader") would have had at least a medical or doctorate degree and probably at least some post-doctoral research experience.

5. To perform this analysis, I have reviewed and understand the contents of the 1994 application. This review included the document titled "Preliminary Amendment" that was filed on 14 November 1994 (hereinafter "the Preliminary Amendment"). I understand that pages 2-19 of the Preliminary Amendment contain text, examples, and claims which are considered part of the 1994 application. I also have reviewed and understand the contents of the 1995 application, including the claims thereof. Exhibit B hereto contains the pending claims of the 1995 application, with claim amendments that the Applicants intend to file with the Patent Office contemporaneously with this declaration.

6. From the facts summarized below, I conclude that the subject matter of claim 1 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Stated another way, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 1 of the 1995 application, at the time that the 1994 application was filed:

A. Claim 31 of the 1994 application recites, "A ligand which specifically binds *to an FLT-4 receptor tyrosine kinase*." (See the Preliminary Amendment at p. 18.) Since claims in a patent application define the invention for which patent applicants seek patent protection, it is absolutely clear to me that the inventors considered an Flt4 ligand to be an aspect of their invention.

B. Claim 1 of the 1995 application recites, "A purified and isolated polypeptide capable of binding with high affinity *to the extracellular domain of Flt4 receptor tyrosine kinase*." Thus, whereas claim 31 of the 1994 application was directed to "a ligand," claim 1 of the 1995 application is directed to "a purified and isolated polypeptide." However, the 1994 application clearly states that the ligand of the invention is a purified protein. (See, e.g., the Preliminary Amendment at p. 15 ("The purified biologically active ligand protein . . . ."); see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or a fragment thereof, which is capable of stimulating the FLT4 receptor....").) Therefore, the "purified and isolated polypeptide" recitations of claim 1 are described in the 1994 application.

C. Whereas claim 31 of the 1994 application was directed to binding "to an FLT-4 receptor tyrosine kinase," claim 1 of the 1995 application specifies that the ligand binds "to *the extracellular domain* of Flt4 receptor tyrosine kinase." However, the 1994 application clearly states that the ligand protein binds to the Flt4 *extracellular domain*. (See, e.g., the Preliminary Amendment at p. 11: "The above experiments prove that the ligand binds to the recombinant FLT4 EC [extracellular] domain.")

Therefore, the recitations in claim 1 regarding binding to the Flt4 *extracellular domain* are described in the 1994 application.

D. Claim 31 of the 1994 application recites that the ligand "specifically binds," whereas claim 1 of the 1995 application is directed to "high affinity" binding. However, the reader would have understood that the "ligand" that "specifically binds" to Flt4 receptor was a high affinity binding partner. For example, the teaching in the 1994 application to purify the ligand using the recombinant FLT4 EC domain in affinity chromatography (see, e.g., the Preliminary Amendment at p. 11 and Example 15) apprises the reader that the ligand is thought to be a high affinity ligand.

Thus, I conclude that the subject matter of claim 1 of the 1995 application is described in claim 31, at pp. 11 and 15 of the Preliminary Amendment, and elsewhere in the 1994 application.

7. I conclude that the subject matter of claim 19 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 19 of the 1995 application is directed to the polypeptide having all of the features recited in claim 1 of the 1995 application, and "further comprising a detectable label." Thus, the only aspect of claim 19 not already discussed above (in paragraph 6) is the inclusion of a detectable label. However, claim 33 of the 1994 patent application recites, "The ligand according to claim 31 comprising a label." (See the Preliminary Amendment at p. 18.) Since claims in a patent application define the invention for which patent applicants seek patent protection, it is absolutely clear to the reader from claims 31 and 33 of the 1994 application that the inventors considered an Flt4 ligand which includes a label to be an aspect of their invention. The property of being "detectable" is understood in the art to be inherent in a "label." (The purpose of a label is to provide a means for detecting the substance that carries the label.) Moreover, this understanding is confirmed by claims 34 and 35 of the 1994 application, which are directed to methods which involve "detecting" the labeled ligand.

(See the Preliminary Amendment at p. 19.) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 19 of the 1995 application, at the time that the 1994 application was filed.

8. I conclude that the subject matter of claim 17 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 17 is similar to claim 1 of the 1995 application and additionally recites that the polypeptide is "purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase." These additional properties are explicitly described in the 1994 application in Examples 12 and 15: Example 12 teaches that conditioned media from the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) produces a soluble ligand for Flt4 that binds to recombinant Flt4 extracellular domain and that can be purified using the Flt4 EC domain in affinity chromatography (see the Preliminary Amendment at pp. 8-11); Example 15 describes such affinity chromatography. (*Id.* at p. 15.) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 17 of the 1995 application, at the time that the 1994 application was filed.

9. I conclude that the subject matter of claim 14 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 14 recites, "A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase." Descriptive support in the 1994 application for "a purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase" is discussed above with respect to claim 1. (See paragraph 6, above.) Example 12 in the 1994 application teaches that the

Flt4 polypeptide ligand stimulates Flt4 tyrosine phosphorylation in mammalian cells that express Flt4 receptor tyrosine kinase. (See the Preliminary Amendment at pp. 8-11; see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or fragment thereof, which is capable of stimulating the Flt4 receptor....").) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 14 of the 1995 application, at the time that the 1994 application was filed.

10. It is fundamental biochemistry that polypeptides are organic chemical compounds, albeit sometimes large and complex ones. Like all organic chemical compounds, polypeptides may be characterized by any of several inherent physical properties, such as molecular formula and molecular weight. Such physical properties are *inherent* characteristics of organic molecules in that they are intrinsic properties of the molecules. Because polypeptides are themselves composed of covalently-bonded chains of smaller organic moieties called amino acids (of which there are about 20 naturally occurring), it is conventional to express the molecular formula of polypeptides as an amino acid sequence. The amino acid sequence of any polypeptide is an inherent property of that polypeptide.

11. Certain claims in the 1995 application recite subject matter that is described in the 1994 application, and also recite certain inherent properties of that subject matter.

A. For example, claims 13 recites a polypeptide having all of the characteristics described in claim 1 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." The subject matter of claim 1 is described in the 1994 application. (See paragraph 6, above.) The approximate 23 kD molecular weight that is recited in claim 13 is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from the PC-3 conditioned medium. (See the

1995 application at pp. 18-19 (teaching that the Flt4 ligand that was affinity purified from PC-3 medium had an apparent molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions).)

B. Claim 15 recites a polypeptide having all of the characteristics described in claim 14 and further recites that the polypeptide comprises "an amino acid sequence set forth in SEQ ID NO: 13." The partial amino acid sequence set forth in SEQ ID NO: 13 of the 1995 application is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from the PC-3 conditioned medium. (See the 1995 application at p. 19, lines 9-19 (teaching that Flt4 ligand that was affinity purified from PC-3 medium had an amino terminal amino acid sequence set forth in SEQ ID NO: 13).)

C. Claim 16 recites a polypeptide having all of the characteristics described in claim 13 and further recites that amino acids 2 through 18 of the polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13. Thus, for the reasons described above with respect to claims 13 and 15 (in Parts A and B), the features recited in claim 16 are inherent properties of an Flt4 ligand that the 1994 application teaches one how to purify from PC-3 conditioned medium.

D. Claim 23 recites a polypeptide having all of the characteristics described in claim 14 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." The subject matter of claim 14 is described in the 1994 application. (See paragraph 9, above.) The approximate 23 kD molecular weight further recited in claim 23 is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from a PC-3 conditioned medium, as discussed in Part A above with respect to claim 13.

The foregoing is not intended to constitute a complete list of those claims which recite inherent properties of an Flt4 ligand described in the 1994 application. For example, the 1995 application teaches a cDNA nucleotide sequence and a

deduced amino acid sequence of a precursor of a 23 kD Flt4 ligand taught in the 1994 application. (See, e.g., 1995 application at p. 5, lines 13-20.) Thus, according to the 1995 application, an inherent property of an Flt4 ligand taught in the 1994 application is that the ligand has an amino acid sequence comprising a portion of SEQ ID NO: 33 that is effective to permit binding to Flt4 receptor tyrosine kinase and stimulate phosphorylation thereof. These properties are recited in several claims of the 1995 application other than those specifically discussed above.

12. The 1994 application teaches the reader how to purify and isolate an Flt4 ligand from conditioned medium of a prostatic cell line, using an affinity chromatography method:

A. Example 12 in the 1994 application teaches the reader how to prepare a conditioned medium comprising an Flt4 ligand by culturing the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) for seven days in F12 medium in the absence of serum, and then clarifying the medium by centrifugation. (See the Preliminary amendment at p. 8.) Example 4 in the 1995 application contains a similar teaching.

B. Example 12 in the 1994 application contains experimental data proving that the PC-3 conditioned medium contains a ligand that is capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase, in cells expressing Flt4 receptor tyrosine kinase. (See the Preliminary Amendment at pp. 8-11.) Moreover, Example 12 in the 1994 application characterizes the Flt4 ligand as a moiety of at least 10,000 molecular weight, and teaches that the medium can be concentrated with a commercially available Centricon-10 concentrator, in order to increase Flt4 ligand activity. (Preliminary Amendment at p. 11.)

C. Example 12 further teaches that treatment of the concentrated PC-3 conditioned medium with Flt4 extracellular domain fragment coupled to Sepharose beads (a solid support) will remove the Flt4 ligand from the conditioned medium. (See the Preliminary Amendment at p. 11 (pretreatment of the concentrated conditioned medium with Flt4EC-

Sepharose abolished the ability of the conditioned medium to stimulate Flt4 phosphorylation).) This teaching provides direct evidence that the ligand of the invention binds to the extracellular domain of Flt4, and thus that the ligand can be purified using the recombinant Flt4 extracellular domain in affinity chromatography.

D. Example 14 of the 1994 application teaches how to make recombinant Flt4 extracellular domain protein to use in an affinity chromatography matrix to purify the Flt4 ligand. (See, e.g., the Preliminary Amendment at p. 13.) Example 3 of the 1995 application contains a similar teaching.

E. Example 15 of the 1994 application teaches how to purify the Flt4 ligand using affinity chromatography procedures. In one of the procedures, the affinity matrix is Flt4 extracellular domain protein that has been cross-linked to CNBr-activated Sepharose 4B (a commercially available solid support that is useful for generating an affinity matrix). The reader in 1994 would have understood that affinity purification involves contacting the ligand-containing solution with the affinity matrix to permit binding between the ligand and the affinity matrix; washing the affinity matrix to remove unbound impurities; and eluting the ligand with an eluting solution. Typically, all fractions removed from the matrix (wash fractions and elution fractions) are assayed to determine in which fractions the ligand of interest has eluted. Example 15 of the 1994 application teaches to use an Flt4 phosphorylation assay to determine which chromatography fractions contained the Flt4 ligand. (See the Preliminary Amendment at p. 15.) The phosphate buffered saline and phosphate buffer wash solutions that were actually used (see the 1995 application at Example 5, p. 18) are typical wash solutions for a protein affinity chromatography. Moreover, the reader would have known that varying parameters such as ionic strength, pH, and the hydrophilic/hydrophobic character of the eluting solutions are conventional methods for eluting a compound of interest from an affinity chromatography column. Thus, the details in Example 15 of the 1994

application enable the reader to purify the Flt4 ligand by affinity chromatography.

F. The 1994 application teaches to subject the Flt4 ligand material that is eluted from the affinity column to further purification, using ion exchange and reverse-phase high pressure chromatography and SDS-polyacrylamide gel electrophoresis. (See the Preliminary Amendment at p. 15.) While the reader would have been able to perform all three of these conventional techniques, it is clear from the results reported in the 1995 application that sufficiently pure Flt4 ligand is obtained (e.g., sufficiently pure for amino acid sequencing) simply with the affinity purification followed by the SDS-PAGE procedure. (See the 1995 application at Example 5, pp. 17-19.) The ion exchange and reverse-phase chromatography were unnecessary.

Thus, the 1994 application teaches the reader how to purify and isolate an Flt4 ligand. The 1995 application describes results of such a purification, thereby demonstrating that the affinity purification method taught in the 1994 application works successfully.

13. The 1994 application teaches several uses for purified Flt4 ligand. These uses include:

A. Isolating a gene encoding the Flt4 ligand by microsequencing the purified ligand to determine a partial amino acid sequence; generating oligonucleotide probes based on the amino acid sequence (See the Preliminary Amendment, Example 15, p. 15; and Example 12, pp. 11-12); using the oligonucleotides as hybridization probes or PCR primers to isolate a ligand-encoding cDNA clone from a cDNA library generated from PC-3 poly-A RNA (*Id.*, Examples 16 and 17A, p. 16);

B. use in an assay system to screen for inhibitors of Flt4 ligand/Flt4 receptor tyrosine kinase interaction (Preliminary Amendment at pp. 6 and 7);

C. regulating the growth, differentiation, and functions of endothelial cells, particularly lymphatic endothelia (Preliminary Amendment at p. 7);

D. generating antibodies against the Flt4 ligand (Preliminary Amendment at p. 7);

E. use in an assay to detect the presence of FLT4 receptor tyrosine kinase (see the Preliminary Amendment at p. 19, claim 35); and

F. use in an assay to detect endothelial cell proliferation (*Id.* , claim 34).

14. With respect to my conclusions in paragraphs 6-13, above, I believe that the reader of ordinary skill in the field in 1994 who reviewed the 1994 application would have reached the same conclusions: that the inventors had possession of a concept of what is now being claimed in the present application. Stated another way, the priority application reasonably would have conveyed to the skilled artisan that the inventors had possession of the Flt4 ligand invention recited in claims of the 1995 application, of how to purify the ligand, and how to use the ligand.

15. I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

June 4, 1997
Date

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A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases

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Angiogenesis, the sprouting of new blood vessels from pre-existing ones, and the permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two known receptors Flt1 (VEGFR-1) and KDR/Flk-1 (VEGFR-2). The Flt4 receptor tyrosine kinase is related to the VEGF receptors, but does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. In this study, we have purified the Flt4 ligand, VEGF-C, and cloned its cDNA from human prostatic carcinoma cells. While VEGF-C is homologous to other members of the VEGF/platelet derived growth factor (PDGF) family, its C-terminal half contains extra cysteine-rich motifs characteristic of a protein component of silk produced by the larval salivary glands of the midge, *Chironomus tentans*. VEGF-C is proteolytically processed, binds Flt4, which we rename as VEGFR-3 and induces tyrosine autophosphorylation of VEGFR-3 and VEGFR-2. In addition, VEGF-C stimulated the migration of bovine capillary endothelial cells in collagen gel. VEGF-C is thus a novel regulator of endothelia, and its effects may extend beyond the lymphatic system, where Flt4 is expressed.

Keywords: angiogenesis/endothelium/growth factor/lymphatic system/VEGF

Introduction

The development of blood vessels from early (*in situ*) differentiating endothelial cells is termed vasculogenesis (Risau and Lemmon, 1988). The formation of the rest of the vascular tree is thought to occur as a result of vascular sprouting from pre-existing vessels, a process called angiogenesis (Folkman, 1995). Vascular endothelial cells can give rise to several types of functionally and morphologically distinct vessels and when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases (Risau, 1995). Angiogenesis also plays a major role in pathological conditions such as diabetic retinopathy, rheumatoid arthritis, psoriasis, cardiovascular diseases and tumour growth and metastasis (Folkman, 1995).

Angiogenesis is regulated by a balance between angiogenic factors and inhibitors which bind to specific receptors on target cells. Five endothelial cell-specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4, Tie and Tek/Tie-2, have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction (for reviews, see Mustonen and Alitalo, 1995; Shibuya, 1995). Targeted mutations inactivating Flt-1, Flk-1, Tie and Tek in mouse embryos have indicated their essential and specific roles in vasculogenesis and angiogenesis at the molecular level (Dumont *et al.*, 1994; Millauer *et al.*, 1994; Fong *et al.*, 1995; Puri *et al.*, 1995; Sato *et al.*, 1995; Shalaby *et al.*, 1995). VEGFR-1 and VEGFR-2 bind VEGF with high affinity (K_d 16 pM and 760 pM, respectively) (de Vries *et al.*, 1992; Terman *et al.*, 1992; Millauer *et al.*, 1993; Waltenberger *et al.*, 1994) and VEGFR-1 also binds the related placenta growth factor (PlGF; K_d ~200 pM) (Maglione *et al.*, 1993; Park *et al.*, 1994), while the ligands for Tie, Tek and Flt4 have not yet been reported.

We report isolation of a novel vascular endothelial growth factor and its cloning from a cDNA library prepared from the human prostatic adenocarcinoma cell line PC-3. We show that the isolated cDNA encodes a protein which is proteolytically processed, secreted to cell culture medium, binds to the extracellular domain of Flt4 and induces tyrosine autophosphorylation of Flt4 and VEGFR-2. VEGF-C also stimulates the migration of endothelial cells in collagen gels.

Results

Identification, purification and N-terminal sequencing of the Flt4 ligand

In order to find a source for the Flt4 ligand, we screened conditioned media (CM) from human tumour cell cultures for their ability to stimulate the Flt4 receptor. Serum-free medium conditioned for 5 days with PC-3 prostatic adenocarcinoma cells was found to stimulate tyrosine phosphorylation of Flt4 expressed in transfected NIH 3T3 cells (Figure 1, lanes 1–3). The stimulating activity was increased upon concentration of CM by ultrafiltration through a 10 kDa cut-off membrane (lanes 2, 3 and 6). Pretreatment with the extracellular domain of Flt4 (Flt4EC) covalently bound to Sepharose completely abolished the ability of CM to stimulate tyrosine phosphorylation of Flt4 (lanes 3–5). No autophosphorylation of Flt4 was detected when transfected cells were treated with purified VEGF or PlGF (Pajusola *et al.*, 1994 and data not shown). These data indicated that the PC-3 cells produce a soluble ligand which binds to the extracellular domain of Flt4 and activates this receptor.

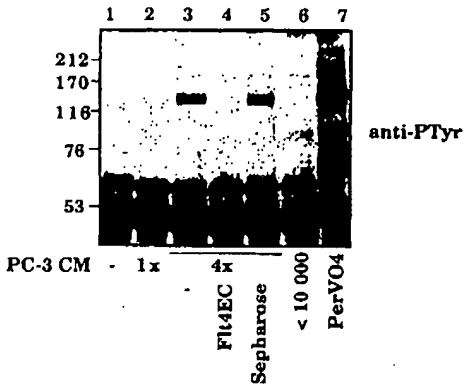


Fig. 1. Identification of the Flt4 ligand from PC-3 cell CM. Flt4-expressing NIH 3T3 cells were incubated with PC-3 cell CM, lysed and the lysates were immunoprecipitated with Flt4-specific antiserum followed by SDS–PAGE, Western blotting and detection using anti-phosphotyrosine (anti-PTyr) antibodies. Lane 1, unconditioned medium. Lane 2 shows weak phosphorylation of a band of 125 kDa upon stimulation with unconcentrated PC-3 CM. The 125 kDa band comigrated with the tyrosine phosphorylated, processed form of the mature Flt4 from pervanadate-treated cells (compare lanes 2 and 7). Lane 3, stimulation with PC-3 CM concentrated 4-fold using Centricon-10 device (Amicon). Lanes 4 and 5, stimulation after treatment of the concentrated PC-3 CM with 30 µl of the recombinant Flt4EC coupled to Sepharose or with unsubstituted Sepharose respectively. Lane 6, Centricon 10 flow-through containing proteins of <10 kDa molecular mass.

The Flt4-stimulating activity was concentrated from PC-3 CM (Figure 2A, lanes 1–3) and used to purify the ligand by affinity chromatography on Flt4EC (lanes 4–11). The Flt4-stimulating material was eluted at pH 2.4 (lanes 8 and 9). Aliquots of the chromatographic fractions were concentrated and subjected to SDS–PAGE under reducing conditions with subsequent silver staining of the gel. As shown in Figure 2B, a major polypeptide having a molecular mass of ~23 kDa (asterisk, lane 6) and a minor one of 32 kDa were detected only in the fractions containing Flt4-stimulating activity, whereas the other polypeptide bands were major components of the starting material. N-terminal amino acid sequence analysis of the 23 kDa band gave the sequence NH_2-XEETIKFAAAHYN-TEILK-COOH.

Cloning of the Flt4 ligand from a PC-3 cDNA library

Degenerate oligonucleotides designed on the basis of the N-terminal sequence of the isolated Flt4 ligand were used as primers in PCR to amplify cDNA encoding the N-terminal peptide from a PC-3 cell cDNA library (see Figure 3A and Materials and methods for details). The product of the expected size was cloned and sequenced and new primers were designed for amplification of the entire 5′ cDNA. The resulting PCR fragment was used as a probe to screen the PC-3 cell cDNA library. The two longest clones of 2.0 and 1.8 kb contained an open reading frame (ORF) of 350 residues shown in Figure 3B, having two possible methionine codons (marked in bold) for translational initiation and a putative secretory signal peptide (underlined) followed by the N-terminal sequence of the purified Flt4 ligand (marked in bold).

Flt4 ligand is a novel member of the PDGF family, VEGF-C

Comparison with the amino acid sequences of growth factors of the VEGF/PDGF family shows that all eight cysteine residues typical for members of this family (Heldin *et al.*, 1993), as well as several other residues are conserved in Flt4 ligand (Figure 3B). Thus, the Flt4 ligand is a novel member of the VEGF family of growth factors, which we have designated VEGF-C. Homologous portions of VEGF-C are ~30% identical to $VEGF_{165}$ (Leung *et al.*, 1989), ~27% to $VEGF\text{-}B_{167}$ (Olofsson *et al.*, 1996), ~25% to PlGF-1 (Maglione *et al.*, 1991) and ~22–24% to PDGF-A and PDGF-B (Betsholtz *et al.*, 1986). However, the VEGF-C polypeptide continues with sequences rich in cysteine residues, some of which can be aligned with the C-terminus of $VEGF_{165}$ as shown in Figure 3B. Interestingly, the C-terminal cysteine residues of VEGF-C occur in repeat units typical for the Balbiani ring 3 protein (BR3P), a major cysteine-rich protein of the larval saliva of the the midge, *Chironomus tentans* (Dignam and Case, 1990; Paulsson *et al.*, 1990). Three repeats, of 24 residues each, are followed by a shorter repeat of 19 residues (Figure 3C), all conforming to the most common type of repeat in BR3P (~40% identity with amino acid sequence 1244–1371) (Paulsson *et al.*, 1990).

Recombinant VEGF-C is proteolytically processed and activates the Flt4 receptor tyrosine kinase

The predicted molecular mass of the secreted polypeptide deduced from the VEGF-C ORF, 35.881 kDa suggests that VEGF-C mRNA may be first translated into a precursor, from which the mature ligand of 23 kDa is derived by proteolytic cleavage. Indeed, a putative precursor polypeptide with an apparent molecular mass of 32 kDa was bound to the Flt4EC affinity matrix from the CM of metabolically labelled cells transfected with a VEGF-C expression vector (Figure 4A). Increased amounts of a 23 kDa receptor binding polypeptide accumulated in the culture medium during a subsequent chase period of 3 h, but not thereafter (lanes 2–4 and data not shown), suggesting that the 23 kDa form is produced by proteolytic processing, which is cell-associated and incomplete, at least in the transiently transfected cells. In non-reducing conditions, higher molecular mass forms were seen, suggesting that the VEGF-C polypeptides can form disulfide-linked dimers (arrows in Figure 4B). The CM of the transfected cells also stimulated Flt4 autophosphorylation (Figure 4C, lanes 1 and 2), but when the CM was pre-absorbed with the Flt4EC, no phosphorylation was obtained (lane 3). On the basis of these results and the above nomenclature, we have renamed Flt4 as VEGFR-3.

Stimulation of VEGFR-2 autophosphorylation by VEGF-C

CM from 293 EBNA cells transfected with the VEGF-C vector was also used to stimulate porcine aortic endothelial (PAE) cells expressing VEGFR-2 (Pajusola *et al.*, 1994; Waltenberger *et al.*, 1994). The cells were lysed and immunoprecipitated using VEGFR-2-specific antiserum (Waltenberger *et al.*, 1994).

The results of the experiment are presented in Figure 5A. A basal level of tyrosine phosphorylation of VEGFR-2 was detected in cells stimulated by CM from the mock-

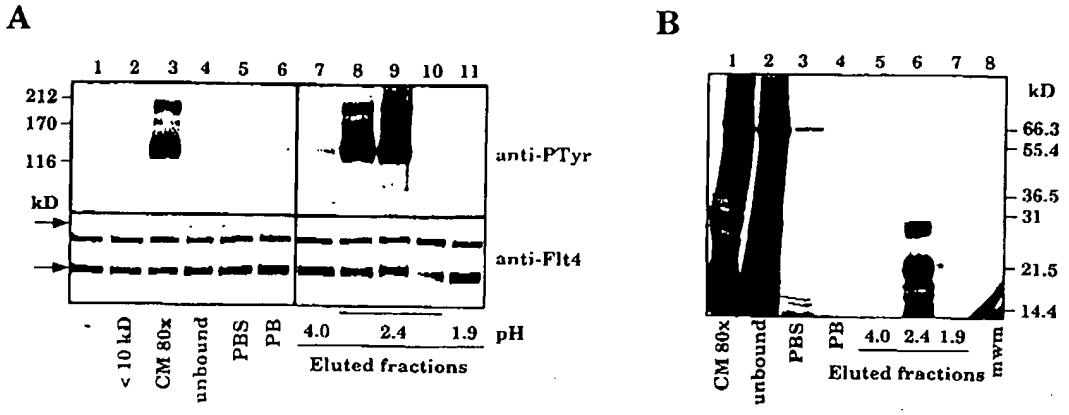


Fig. 2. Purification of the Flt4 ligand. (A) Flt4-expressing cells were treated with non-conditioned medium (lane 1), PC-3 cell CM or with different chromatographic fractions and Flt4 was immunoprecipitated and analysed in SDS–PAGE followed by Western blotting and detection with PTyr antibodies or Flt4-specific antiserum and the ECL method. The phosphorylated unprocessed 195 kDa and proteolytically processed 125 kDa forms of Flt4 (Pajusola *et al.*, 1994) are marked by arrows. Note that the (presumably intracellular) 175 kDa precursor of Flt4 is not phosphorylated upon stimulation. (B) Aliquots of the chromatographic fractions were concentrated and subjected to SDS–PAGE under reducing conditions with subsequent silver staining of the gel. The designations of the lanes are as follows: <10 kDa and CM 80×, filtrate and retained fractions, respectively, obtained after concentration of CM by ultrafiltration through a 10 kDa cut-off membrane; unbound, CM after absorption with FLT4EC; PBS and PB, washes of the affinity matrix with phosphate buffered saline and phosphate buffer pH 6.8, respectively; 4.0, 2.4 and 1.9, fractions eluted from the affinity matrix at indicated pHs; mwm, molecular mass markers.

transfected cells. A further concentration of this medium resulted in only a slight enhancement of VEGFR-2 phosphorylation (lanes 1 and 2). CM containing recombinant VEGF-C stimulated tyrosine autophosphorylation of VEGFR-2 and the intensity of the autophosphorylated polypeptide band was increased upon concentration of the VEGF-C CM (lanes 3–5). Furthermore, the stimulating effect was abolished after pretreatment of the medium with the Flt4EC affinity matrix (compare lanes 1, 5 and 6). The maximal effect of VEGF-C in this assay was comparable with the effect of recombinant VEGF added to the unconditioned medium at a concentration of 50 ng/ml (lane 8). Pretreatment of the medium containing VEGF with Flt4EC did not abolish its stimulating effect on VEGFR-2 (compare lanes 7 and 8). These results suggest that the VEGF-C expression vector encodes a ligand not only for VEGFR-3, but also for VEGFR-2.

In order to further confirm that the stimulating effect of VEGF-C on tyrosine phosphorylation of VEGFR-3 and VEGFR-2 was receptor-specific, we analysed the effect of VEGF-C on tyrosine phosphorylation of PDGF receptor β (PDGFR-β) which is abundantly expressed on fibroblastic cells. As can be seen from Figure 5B, a weak tyrosine phosphorylation of PDGFR-β was detected upon stimulation of Flt4-expressing NIH 3T3 cells with CM from the mock-transfected cells (compare lanes 1 and 2). A similar low level of PDGFR-β phosphorylation was observed when the cells were incubated with CM from the VEGF-C-transfected cells, with or without prior treatment with Flt4EC (lanes 3 and 4). In contrast, the addition of 50 ng/ml of PDGF-BB induced a prominent tyrosine autophosphorylation of PDGFR-β (lane 5).

VEGF-C stimulates endothelial cell migration in collagen gels

CM from cell cultures transfected with the VEGF-C expression vector was placed in a well which was made in collagen gel and used to stimulate the migration of bovine capillary endothelial (BCE) cells in the three-dimensional collagen gel assay described in Materials and methods. After 6 days of treatment, the cultures were stained and cells at different distances outside of the original ring of attachment were counted using fluorescent nuclear staining and detection with a fluorescence microscope equipped with a grid. A comparison of the numbers of migrating cells in successive 0.5×0.5 mm areas is shown in Figure 6A. As can be seen from the columns, VEGF-C-containing CM stimulated cell migration more than medium conditioned by the non-transfected or mock-transfected cells but less than medium from cells transfected with a VEGF expression vector. An example of typical phase contrast and fluorescent microscopic fields of cultures stimulated with medium from mock-transfected or VEGF-C-transfected cells is shown in Figure 6B. Daily addition of 1 ng of FGF2 into the wells resulted in the migration of approximately twice the number of cells when compared with the stimulation by CM from VEGF-transfected cells (data not shown).

VEGF-C is expressed in multiple tissues

Northern blotting and hybridization analysis showed that a 2.4 VEGF-C mRNA is present in the HT-1080 fibrosarcoma and PC-3 prostatic adenocarcinoma cell lines (Figure 7A). The 2.4 kb RNA and smaller amounts of a 2.0 kb mRNA were seen in multiple human tissues, most prominently in the heart, placenta, muscle, ovary and small intestine (Figure 7B). Very little VEGF-C RNA was seen in the brain, liver or thymus and peripheral blood leukocytes (pbl) appeared negative.

Discussion

Our results show that VEGFR-3 transmits signals for a novel growth factor. This conclusion is based on the specific binding of VEGF-C to recombinant Flt4EC protein and the induction of VEGFR-3 autophosphorylation by medium from VEGF-C-transfected cells. In contrast,

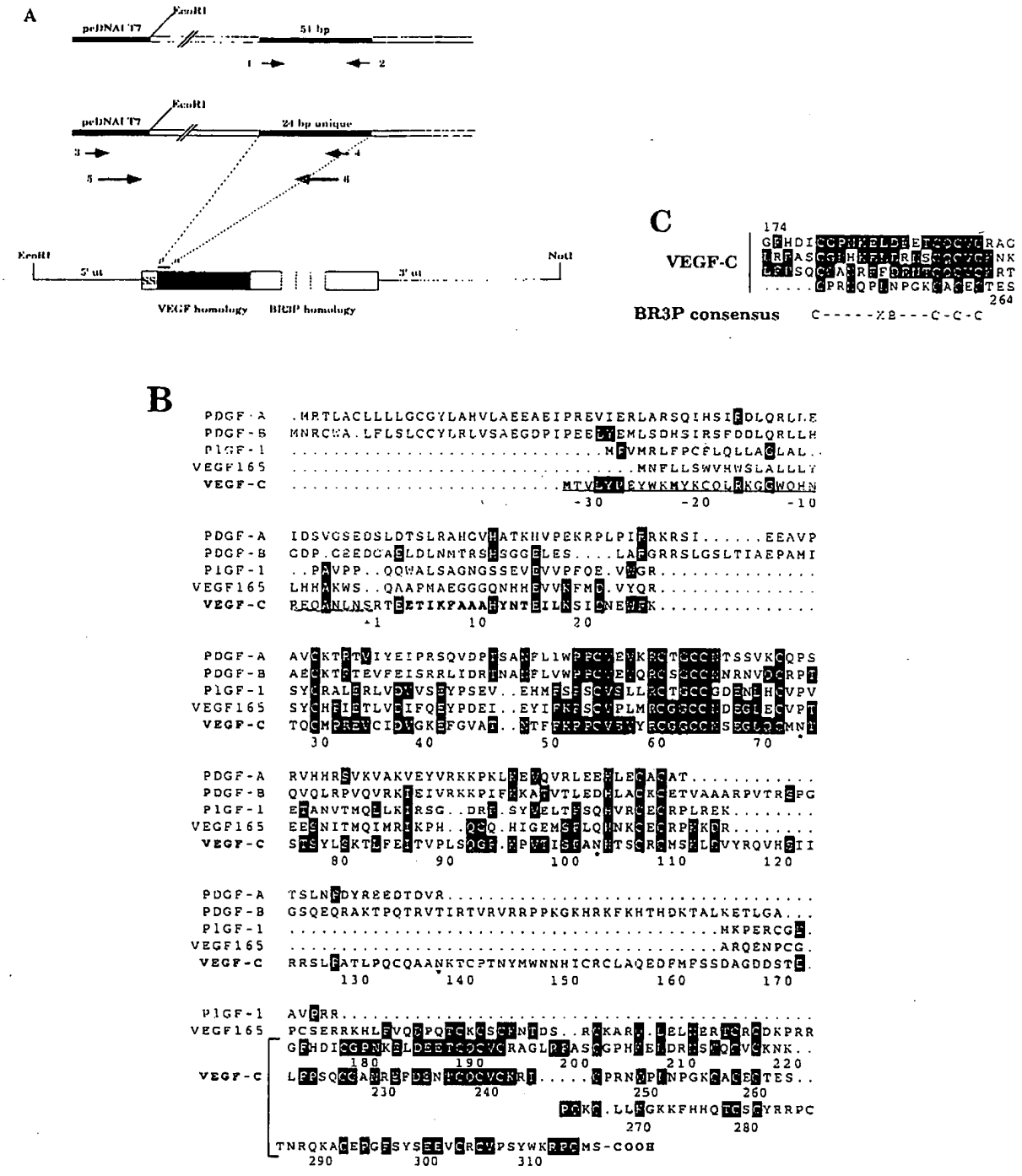


Fig. 3. Cloning and analysis of the Flt4 ligand (VEGF-C). (A) Schematic illustration of the PCR cloning of Flt4 ligand cDNA from PC-3 cDNA library. The primers and conditions used are detailed in Materials and methods. (B) Comparison of the amino acid sequence of VEGF-C with other growth factors of the VEGF/PDGF family (Betsholtz *et al.*, 1986; Leung *et al.*, 1989; Maglione *et al.*, 1991). VEGF-C amino acid residues are numbered beginning from the N-terminus after cleavage of the signal sequence. The PileUp program of Genetics Computer Group was used for alignment of the VEGF-homologous domains. The C-terminal motifs were aligned on basis of the pattern of cysteine residues. Three putative N-linked glycosylation sites (N-X-S/T) have been marked with asterisks. (C) Alignment of the repeated C-terminal motifs of VEGF-C with the consensus sequence of BR3P. B = D or N residue. X = non-polar or tyrosyl residue.

VEGF or PlGF did not show specific binding to VEGFR-3 or induce its autophosphorylation (Pajusola *et al.*, 1994).

Interestingly, the VEGF-C ORF is 350 amino acid residues long and our N-terminal sequence analysis confirmed that its putative signal sequence is removed before secretion. Glutamic acid was the second residue obtained in the N-terminal sequence analysis of the isolated protein, while the first residue could not be determined. According

to the deduced amino acid sequence of the VEGF-C cDNA this first residue is threonine. However, on the basis of the consensus residues surrounding signal sequence cleavage sites (von Heijne, 1986), the first residue following the signal sequence would be arginine, which may have been removed from the polypeptide after an additional proteolytic cleavage between arginine and threonine residues (see Figure 3B).

A major part of the difference in the observed molecular mass of the purified and recombinant VEGF-C and the deduced molecular mass of the VEGF-C encoded by the ORF may be due to proteolytic removal of sequences in the C-terminal region of the latter. Proteolytic processing of the VEGF-C precursor may occur at more than one cleavage site because the molecular mass of the recombinant secreted ligand, 32 kDa, was also less than the deduced molecular mass of the VEGF-C ORF without the signal peptide (see Figure 4A). By extrapolation from studies of the structure of PDGF (Heldin *et al.*, 1993), one can speculate that the region critical for receptor binding and activation by VEGF-C is contained within the first 180 or so amino acid residues of VEGF-C. Thus, the 23 kDa polypeptide binding VEGFR-3 is likely to represent the VEGF-homologous domain, which may be glycosylated at three putative N-linked glycosylation sites identified in the deduced VEGF-C amino acid sequence.

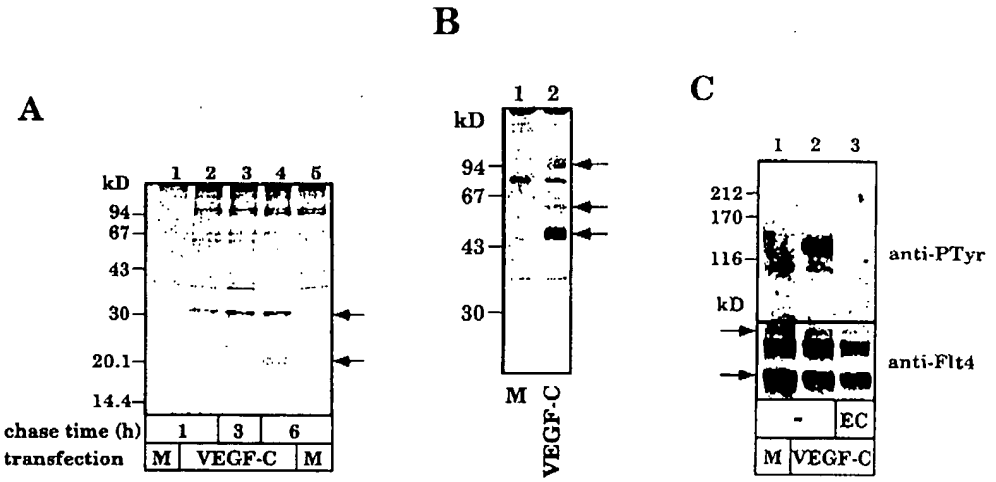


Fig. 4. Identification, dimerization and activity of recombinant VEGF-C. (A) Pulse–chase analysis of VEGF-C secreted by transfected cells. 293 EBNA cells were metabolically labelled using ^{35}S-labelled methionine and cysteine mixture for 2 h and then chased in non-radioactive medium for the indicated periods of time. The medium was collected and VEGF-C was bound to Flt4EC–Sepharose followed by alkylation, SDS–PAGE and autoradiography. M = mock-transfected cells. Arrows indicate the 32 kDa and 23 kDa polypeptides of secreted VEGF-C. (B) VEGF-C isolated using Flt4EC–Sepharose after a 4 h continuous metabolic labelling was analysed under non-reducing conditions. The arrows indicate putative dimeric forms. (C) Stimulation of VEGFR-3 autophosphorylation by VEGF-C. NIH 3T3 cells expressing VEGFR-3 were stimulated with medium conditioned by cells transfected with VEGF-C cDNA. The medium was either untreated (lane 2) or treated with Flt4 EC (lane 3). Arrows indicate the phosphorylated forms of Flt4 (see the legend of Figure 2A).

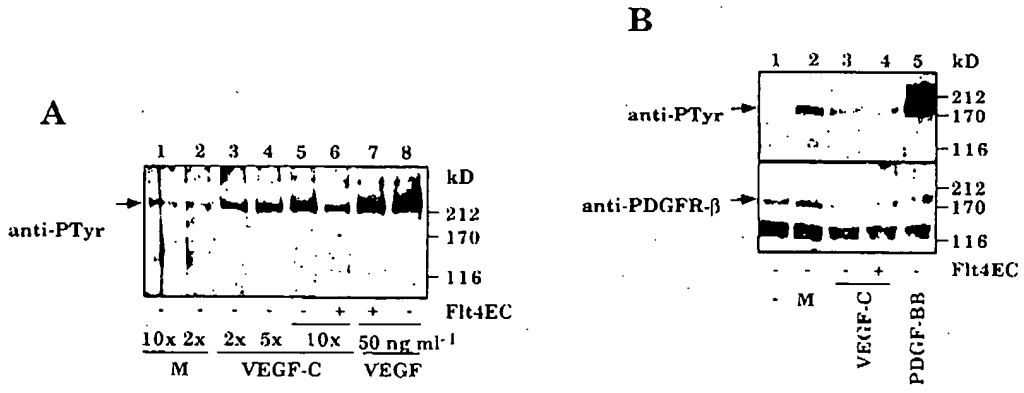
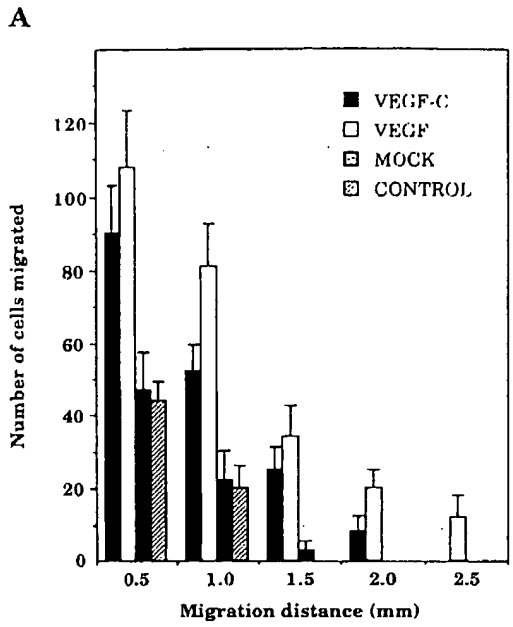


Fig. 5. VEGF-C stimulates autophosphorylation of VEGFR-2 (KDR) but has no effect on PDGFR-β phosphorylation. (A) PAE cells expressing VEGFR-2 were stimulated with 10- or 2-fold concentrated medium from mock-transfected 293 EBNA cells (lanes 1 and 2), or with 2-, 5- or 10-fold concentrated medium from 293-EBNA cell cultures expressing the recombinant VEGF-C (lanes 3–6). VEGFR-2 was immunoprecipitated with specific antibodies and analysed by SDS–PAGE and Western blotting using phosphotyrosine antibodies. For comparison, the stimulations were carried out with non-conditioned medium containing 50 ng/ml of purified recombinant VEGF (lanes 7 and 8). Lanes 6 and 7 show stimulation with VEGF-C or VEGF containing media pretreated with Flt4EC. (B) Flt4-expressing NIH 3T3 cells were stimulated with non-conditioned medium (lane 1), 5-fold concentrated CM from mock-transfected (lane 2) or VEGF-C-transfected (lanes 3 and 4) cells, or with non-conditioned medium containing 50 ng/ml of recombinant human PDGF-BB (lane 5). Medium containing VEGF-C was also pretreated with recombinant Flt4EC (lane 4). PDGFR-β was immunoprecipitated with specific antibodies and analysed by SDS–PAGE and Western blotting using phosphotyrosine antibodies with subsequent stripping and reprobing of the membrane with antibodies specific for PDGFR-β.



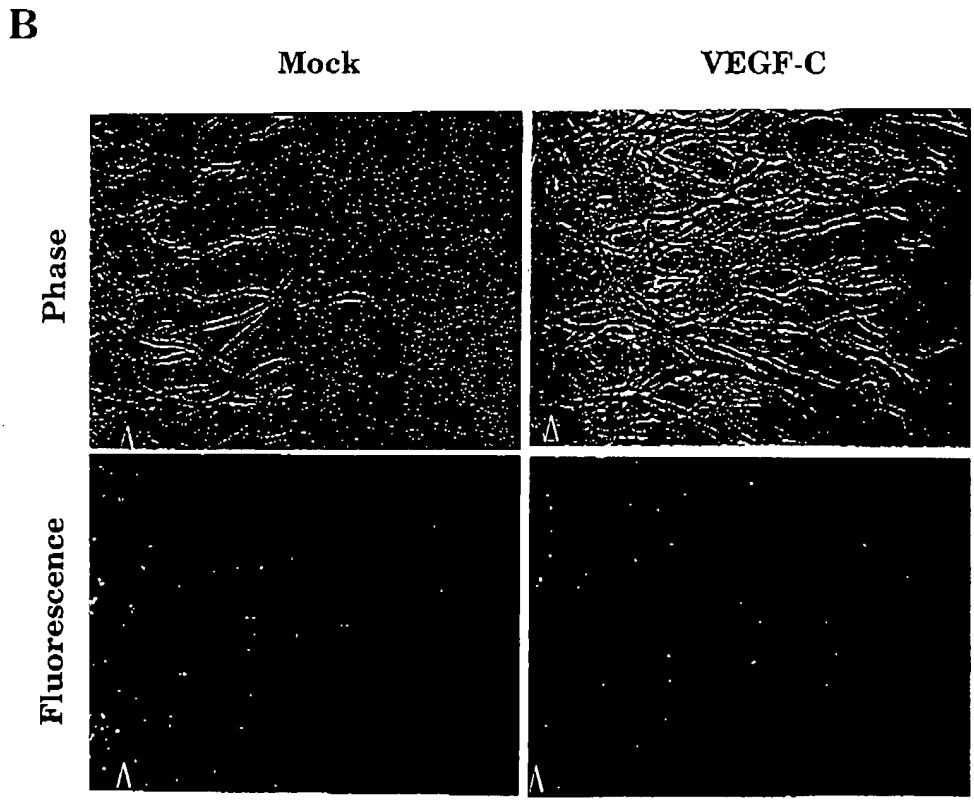


Fig. 6. VEGF-C stimulates endothelial cell migration in a three-dimensional collagen gel assay. (A) The diagram shows a comparison of the number of cells migrating at different distances from the original area of attachment towards wells containing media conditioned by the non-transfected (control) or transfected (mock; VEGF-C; VEGF) cells. 6 days after addition of the media. The number of cells migrating out from the original ring of attachment was counted in five adjacent 0.5×0.5 mm squares using a microscope ocular lens grid and 10× magnification. Cells migrating further than 0.5 mm were counted in a similar way by moving the grid in 0.5 mm steps. The experiments were carried out twice in duplicate with similar results, and medium values from the one of the experiments are presented with the standard error bars. (B) Phase-contrast microscopy and fluorescent microscopy of the nuclear staining of BCE cells migrating towards the wells containing media conditioned by the mock-transfected cells or by VEGF-C-transfected cells. The areas shown are ~1×1.5 mm and arrows indicate the borders of the original ring of attachment.

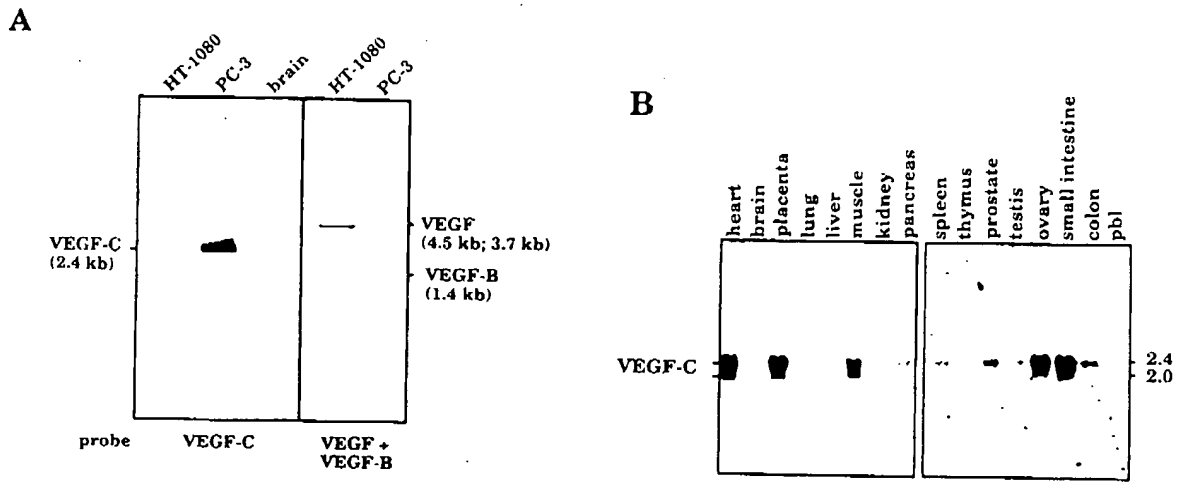


Fig. 7. Expression of VEGF-C mRNA in tumour cell lines and in human adult tissues. Northern blots containing 8 μg of isolated poly(A)$^+$ RNA from HT-1080 and PC-3 human tumour cells (A) and multiple human tissues (B, blot from Clontech) were probed with radioactively labelled insert of the 2.0 kb VEGF-C cDNA clone. Shown in (A) are also the 4.5 kb and 3.7 kb mRNA signals for VEGF and the 1.4 kb signal for VEGF-B (Olofsson *et al.*, 1996) in the same samples. Note that the tumour cell lines contain mainly mRNA of the 2.4 kb form.

The C-terminal amino acid sequences, which increase the length of the VEGF-C polypeptide in comparison with other ligands of this family show a pattern of spacing of cysteine residues reminiscent of the BR3P sequence (Dignam and Case, 1990; Paulsson *et al.*, 1990). This novel C-terminal silk-protein-like structural motif of VEGF-C may fold into an independent domain, which, on the basis of the considerations above, is at least partially cleaved off after biosynthesis. Interestingly, at least one cysteine motif of the BR3P type is also found in the C-terminus of VEGF. In our experiments both the putative precursor and cleaved ligand were detected in the cell culture medium, although processing was apparently cell-associated on the basis of the pulse–chase experiments. The determination of the N-terminal sequence of the isolated C-terminal fragment will allow the identification of the proteolytic processing site. On the other hand, the generation of antibodies against different parts of the VEGF-C molecule will allow the exact determination of the precursor–product relationship and ratio, their cellular distribution and the kinetics of processing and secretion.

We have recently cloned another factor structurally homologous to VEGF, designated accordingly as VEGF-B (Olofsson *et al.*, 1996). Both of these factors share a conserved pattern of eight cysteine residues, which may participate in the formation of intra- and interchain disulfide bonds creating an antiparallel dimeric biologically active molecule, similar to PDGF (Andersson *et al.*, 1992; Oefner *et al.*, 1992). Mutational analysis of the cysteine residues involved in the interchain disulfide bridges have shown that, in contrast to PDGF, VEGF dimers need to be held together by these covalent interactions in order to maintain biological activity (Pötgens *et al.*, 1994). Putative dimers were evident in the analysis of VEGF-C under non-reducing conditions. It will be interesting to analyse these possible dimerization patterns of VEGF-C, but this is made technically difficult by the presence of precursor and processed forms and the high cysteine content of VEGF-C, which causes anomalous migration in gel electrophoresis under non-reducing conditions.

VEGFR-3, which thus distinguishes between VEGF and VEGF-C, is closely related in structure to VEGFR-1 and VEGFR-2 (Pajusola *et al.*, 1992; Finnerty *et al.*, 1993; Galland *et al.*, 1993). However, the mature form of VEGFR-3 differs from the two other VEGFRs in that it is proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (Pajusola *et al.*, 1994). Another difference is that the 4.5 and 5.8 kb VEGFR-3 mRNAs encode polypeptides differing in their C-termini and apparently in their signalling properties due to the use of alternative 3′ exons (Pajusola *et al.*, 1993; Borg *et al.*, 1995).

Besides VEGFR-3, VEGFR-2 tyrosine kinase also was shown to be activated in response to VEGF-C. VEGFR-2-mediated signals cause striking changes in the morphology, actin reorganization and membrane ruffling of PAE cells overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity (Waltenberger *et al.*, 1994). Similarly, the receptor chimera CSF-1R/VEGFR-3 was mitogenic when ectopically expressed in NIH 3T3 fibroblastic cells, but not in PAE cells (Pajusola *et al.*, 1994). Consistent with such results, the bovine capillary endothelial cells, which express VEGFR-2 mRNA but very little or no VEGFR-1 or VEGFR-3 mRNAs (our unpublished data), showed enhanced migration when stimulated with VEGF-C. Light microscopy of the BCE cell cultures in collagen gel also suggested that VEGF-C stimulated the proliferation of these cells, but such data must be confirmed by more detailed analyses of cell proliferation and survival in the presence and absence of specific factors. The existing data thus indicate that the VEGF ligands and receptors show a great specificity in their signalling, which may be cell type dependent.

The expression pattern of the VEGFR-3 (Kaipainen *et al.*, 1995) suggests that VEGF-C may function in the

formation of the venous and lymphatic vascular systems during embryogenesis. Constitutive expression of VEGF-C in adult tissues further suggests that this gene product is also involved in the maintenance of the differentiated functions of the lymphatic endothelium where VEGFR-3 is expressed (Kaipainen *et al.*, 1995). Lymphatic capillaries do not have well formed basal laminae and an interesting possibility remains that the silk-like BR3P motif is involved in producing a supramolecular structure which could regulate the availability of VEGF-C in tissues. However, VEGF-C also activates VEGFR-2, which is abundant in proliferating endothelial cells of vascular sprouts and branching vessels of embryonic tissues, but decreased in adult tissues (Millauer *et al.*, 1993). These data have suggested that VEGFR-2 is a major regulator of vasculogenesis and angiogenesis. VEGF-C may thus have a unique effect in lymphatic endothelium and a more redundant function shared with VEGF in angiogenesis and possibly permeability regulation of several types of endothelia.

Taken together these results show an increased complexity of signalling in the vascular endothelium. They reinforce the concept that when organs differentiate and begin to perform their specific functions, the phenotypic heterogeneity of endothelial cells increases in several types of functionally and morphologically distinct vessels. However, upon suitable angiogenic stimuli, endothelial cells can re-enter the cell cycle, migrate, withdraw from the cell cycle and subsequently differentiate again to form new vessels which are functionally adapted to their tissue environment. This process of angiogenesis, concurrent with tissue development and regeneration, depends on the tightly controlled balance between positive and negative signals for endothelial cell proliferation, migration, differentiation and survival. Previously identified growth factors promoting angiogenesis include the fibroblast growth factors, hepatocyte growth factor/scatter factor, PDGF and TGF-α (for references, see Folkman, 1995; Friesel and Maciag, 1995; Mustonen and Alitalo, 1995). However, VEGF, which was identified ~10 years ago (Senger *et al.*, 1983), has been the only growth factor relatively specific for endothelial cells. Thus the newly identified factors VEGF-B (Olofsson *et al.*, 1996) and VEGF-C (the present data) increase our understanding of the complexity of the specific and redundant positive signals for endothelial cells involved in vasculogenesis, angiogenesis, permeability and perhaps other endothelial functions.

Materials and methods

Cell culture

Human prostatic adenocarcinoma PC-3 cells (American Type Culture Collection CRL 1435) were cultured in Ham's F12 medium supplemented with 7% fetal calf serum (FCS); 293 EBNA cells (Invitrogen) and NIH 3T3-Flt4 cells (Pajusola *et al.*, 1993) in DMEM–10% FCS; PAE-KDR cells (Waltenberger *et al.*, 1994) in Ham's F12 medium–10% FCS. BCE cells (Folkman *et al.*, 1979) were cultured as described in Pertovaara *et al.* (1994). After reaching confluence the monolayers of PC-3 cells were cultured for 5 days in Ham's F12 medium without FCS. CM was then collected, clarified by centrifugation at 10 000 *g* and used for purification of VEGF-C.

Analysis of stimulation of the receptors

Confluent NIH 3T3-Flt4 cells or PAE-KDR cells were starved overnight in DMEM or Ham's F12 media, respectively, supplemented with 0.2% BSA and then incubated for 5 min with the analysed media. Recombinant human VEGF (R&D Systems) and PDGF-BB were used as control stimulating agents. The cells were washed twice with ice-cold Tris-buffered saline (TBS) containing 100 μM sodium orthovanadate and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 U/ml aprotinin and 1 mM sodium orthovanadate. The lysates were sonicated, clarified by centrifugation at 16 000 *g* for 20 min and incubated for 3–6 h on ice with 3–5 μl of antisera specific for Flt4 (Pajusola *et al.*, 1993), KDR or PDGFR-β (Claesson-Welsh *et al.*, 1989; Waltenberger *et al.*, 1994). Recombinant human PDGF-BB as well as antisera specific for KDR and PDGFR-β were kindly provided by Dr Lena Claesson-Welsh. Immunoprecipitates were bound to protein A–Sepharose, washed three times with TBS containing 1 mM PMSF and 1 mM sodium orthovanadate, twice with 10 mM Tris–HCl pH 7.4 and subjected to SDS–PAGE in a 7% gel (Laemmli, 1970). Polypeptides were transferred to nitrocellulose by Western blotting and analysed using PY20 phosphotyrosine-specific monoclonal antibodies (Transduction Laboratories) or receptor-specific antiserum and the ECL method (Amersham).

Production and purification of baculoviral Flt4EC protein

The segment of Flt4 cDNA (GenBank Accession Number X68203) encoding EC was amplified in PCR using primers which encoded six additional C-terminal His residues followed by a stop codon, and added *Bam*HI sites at both ends. The amplified fragment was then cloned into the *Bam*HI site in the pVTBac plasmid (Tessier *et al.*, 1991), which was used to generate a Flt4EC baculovirus. The Flt4EC protein was purified from the culture medium of baculovirus-infected High-Five cells (Invitrogen) by Ni-NTA affinity chromatography (Qiagen) and coupled to CNBr-activated Sepharose 4B (Pharmacia; 5 mg of Flt4 EC/ml Sepharose resin).

Isolation and N-terminal sequence analysis of VEGF-C

Eight litres of PC-3 CM was concentrated 80-fold using a 10 kDa cut-off ultrafiltration membrane (Filtron Technology Corporation) and incubated with the recombinant Flt4EC–Sepharose affinity matrix. The affinity matrix was washed successively with PBS and 10 mM PB (pH 6.8) and the bound material was eluted step-wise with 100 mM glycine–HCl, successive eluates having pHs of 4.0, 2.4 and 1.9. Eluates were collected in tubes containing 1/4 volume of 1 M Na-phosphate pH 8.0, dialysed against 1 mM Tris–HCl pH 7.5 and the aliquots were analysed for their ability to stimulate tyrosine phosphorylation of VEGFR-3.

Two fractions eluted from the affinity matrix at pH 2.4 were combined, vacuum dried and subjected to SDS–PAGE in a 12.5% gel. The proteins from the gel were then electroblotted to Immibilon-P transfer membrane (Millipore) and visualized by staining of the blot with Coomassie blue R-250. The region containing only the stained 23 kDa band was cut from the blot and subjected to N-terminal amino acid sequence analysis in a Prosite Protein Sequencing System (Applied Biosystems).

Cloning of VEGF-C from a PC-3 cell expression library

Six micrograms of poly(A)$^+$ RNA derived from PC-3 cells was used to prepare an oligo(dT)-primed cDNA library using the Librarian kit of Invitrogen. The first PCR was carried out using 1 μg of DNA from the library and the primers marked in the Figure 3A: 1. 5′-GCAGARG-ARACNATHAA-3′ (wherein R is A or G, N is A, G, C or T and H is A, C or T) and 2. 5′-GCAYTTNARDATYTCNGT-3′ (wherein Y is C or T and D is A, G or T). Two successive PCRs were carried out using 1 U per reaction of DynaZyme (Finnzymes), at an extension temperature of 72°C for 43 cycles, the first three cycles at annealing temperature 33°C for 2 min and the remaining ones at 42°C for 1 min. A band of the expected size (57 bp) was re-amplified for 30 cycles in the latter conditions, cloned into a pCRII vector (Invitrogen) and sequenced. All six clones analysed contained the sequence encoding the expected N-terminal peptide (although they were later found also to have mismatches with the final sequence of the cloned cDNA). Based on the unique nucleotide sequence obtained two pairs of nested primers were designed to amplify the complete 5′-end of the cDNA. The primers were 3. 5′-TAATACGACTCACTATAGGG-3′ and 4. 5′-TCNGTGTTGTAGTGTG-CTG-3′, the former corresponding to the pcDNAI vector used for construction of the library. 'Touchdown' PCR was used (Don *et al.*, 1991). The annealing temperature of the two first cycles was 62°C and subsequently 1°C less in steps of two cycles until a final temperature of 53°C was reached, at which temperature 16 additional cycles were carried out. Annealing time was 1 min and extension at 72°C for 1 min. The products of the first amplification (1 μl of a 1:100 dilution in water) were used in the second amplification reaction employing the nested

primers 5. 5'-TCACTATAGGGAGACCCAAGC-3' and 6. 5'-GTTGTA-GTGTGCTGCAGCGAATTT-3'. The annealing temperature was decreased in Touchdown PCR from 72°C to 66°C and continued with 18 additional cycles at 66°C. The annealing time was 1 min and extension at 72°C for 2 min. A product of ~220 bp was cloned into the pCR II vector, sequenced and found to contain the 5'-end of the VEGFR-3 ligand cDNA. This fragment was digested with *Eco*RI, and the resulting 153 bp fragment was labelled with [^{32}P]dCTP and used as a probe for hybridization screening of the amplified PC-3 cell cDNA library.

Expression and detection of recombinant VEGF-C

The 2.0 kb insert of the VEGF-C clone in pcDNA1 vector was cut out from the vector using *Hin*dIII and *Not*I restriction enzymes and ligated into the corresponding sites in the pREP7 expression vector (Invitrogen). The resulting plasmid was transfected into 293 EBNA cells using a calcium phosphate precipitation method. An equivalent amount of the pREP7 plasmid without insert was used in mock transfections. The culture medium was changed to DMEM–0.2% BSA 48–72 h after transfection and after an additional 24 h this medium was collected, clarified by centrifugation and used for studies of the effects of VEGF-C. In some cases CM was concentrated using Centriprep-10 devices (Amicon).

Metabolic labelling of 293 EBNA cells transfected with the VEGF-C construct was carried out by addition of 100 μCi/ml of Pro-mix™ L-[^{35}S] *in vitro* cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After 2 h the cell layers were washed twice with PBS and the medium was then replaced with DMEM–0.2% BSA. After 1, 3, 6, 12 and 24 h of subsequent incubation the culture medium was collected, clarified by centrifugation, concentrated and VEGF-C was bound to 30 μl of a slur of Flt4EC–Sepharose overnight at 4°C, followed by three washes in PBS, two washes in 20 mM Tris–HCl pH 7.5, alkylation, SDS–PAGE and autoradiography.

Endothelial cell migration in three-dimensional collagen gel

The collagen gels were prepared by mixing type I collagen stock solution (5 mg/ml in 1 mM HCl) with an equal volume of 2× MEM and two volumes of MEM containing 10% newborn calf serum (NCS) to give a final collagen concentration of 1.25 mg/ml. The tissue culture plates (5 cm diameter) were coated with ~1 mm thick layer of the solution, which was allowed to polymerize at 37°C. BCE cells were seeded on top of this layer. For the migration assays, the cells were allowed to attach inside a plastic ring (1 cm diameter) placed on top of the first collagen layer. After 30 min the ring was removed and unattached cells were rinsed away. A second layer of collagen and a layer of growth medium (5% NCS) solidified by 0.75% low melting point agar (FMC BioProducts, Rockland, ME) were added. A well (3 mm diameter) was punched through all the layers on both sides of the cell spot at a distance of 4 mm and the media were daily pipetted into the wells. Photomicrographs of the cells migrating out from the spot edge were taken after 6 days through an Olympus CK 2 inverted microscope equipped with phase-contrast optics. The migrating cells were counted after nuclear staining with the fluorescent dye bisbenzimide (1 μg/ml, Hoechst 33258, Sigma).

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Proteolytic processing regulates receptor specificity and activity of VEGF-C

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The recently identified vascular endothelial growth factor C (VEGF-C) belongs to the platelet-derived growth factor (PDGF)/VEGF family of growth factors and is a ligand for the endothelial-specific receptor tyrosine kinases VEGFR-3 and VEGFR-2. The VEGF homology domain spans only about one-third of the cysteine-rich VEGF-C precursor. Here we have analysed the role of post-translational processing in VEGF-C secretion and function, as well as the structure of the mature VEGF-C. The stepwise proteolytic processing of VEGF-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2. Recombinant 'mature' VEGF-C made in yeast bound VEGFR-3 (K_D = 135 pM) and VEGFR-2 (K_D = 410 pM) and activated these receptors. Like VEGF, mature VEGF-C increased vascular permeability, as well as the migration and proliferation of endothelial cells. Unlike other members of the PDGF/VEGF family, mature VEGF-C formed mostly non-covalent homodimers. These data implicate proteolytic processing as a regulator of VEGF-C activity, and reveal novel structure–function relationships in the PDGF/VEGF family.

Keywords: angiogenesis/growth factor/proteolytic processing/VEGF/VEGF-C

Introduction

Angiogenesis, the formation of blood vessels by sprouting from pre-existing ones, is regulated by a balance between positive and negative regulators (Hanahan and Folkman, 1996). Vascular endothelial growth factor (VEGF) belongs to the platelet-derived growth factor (PDGF)/VEGF family and is a major inducer of angiogenesis in normal and pathological conditions (Dvorak *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Ferrara, 1997). The biological effects of VEGF are largely specific for endothelial cells and include stimulation of their proliferation, migration and tube formation, and regulation of vascular permeability (Dvorak *et al.*, 1995; Klagsbrun and D'Amore, 1996; Ferrara, 1997). Another growth factor of the VEGF family, placenta growth factor (PlGF), is expressed predominantly in the placenta; it has minimal angiogenic activity, but is able to heterodimerize with and to modulate the effects of VEGF (Maglione *et al.*, 1991; Park *et al.*, 1994; DiSalvo, 1995; Cao *et al.*, 1996).

VEGF binds to and induces biological responses via two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk1/KDR), expressed mainly in endothelial cells (see Mustonen and Alitalo, 1995; Shibuya, 1995 for references). PlGF is exclusively a ligand for VEGFR-1 (Park *et al.*, 1994). VEGFR-1 and VEGFR-2 have seven immunoglobulin (Ig)-like loops in the extracellular domain (EC), a single transmembrane region and a tyrosine kinase domain, interrupted by an insert of 60–70 amno acid residues (de Vries *et al.*, 1992; Terman *et al.*, 1992; Shibuya, 1995).

Three novel growth factors strikingly similar to VEGF and PlGF have been identified recently. These factors are the VEGF-B/VEGF-related factor (VRF) (Grimmond *et al.*, 1996; Olofsson *et al.*, 1996a), VEGF-C/VEGF-related protein (VRP) (Joukov *et al.*, 1996; Lee *et al.*, 1996) and c-*fos*-induced growth factor (FIGF) (Orlandini *et al.*, 1996). VEGF-B is most closely related to VEGF and is able to form heterodimers with it (Olofsson *et al.*, 1996a,b). VEGF-C and FIGF are similar in that both have N- and C-terminal extensions flanking a VEGF homology domain. Their C-terminal propeptides contain tandemly repeated motifs with a spacing of cysteine residues typical of Balbiani ring 3 protein (BR3P) (Joukov *et al.*, 1996; Kukk *et al.*, 1996; Lee *et al.*, 1996; Orlandini *et al.*, 1996). Thus, VEGF-C and FIGF comprise a novel subgroup of the PDGF/VEGF family.

The receptors for VEGF-B and FIGF have not yet been identified, while VEGF-C is a ligand for two receptors, VEGFR-3 (Flt4) (Joukov *et al.*, 1996; Lee *et al.*, 1996) and VEGFR-2 (Joukov *et al.*, 1996). VEGFR-3 differs from the two other VEGFRs by being proteolytically cleaved in the extracellular domain into two disulfide-linked polypeptides (Aprelikova *et al.*, 1992; Pajusola *et al.*, 1992, 1993; Galland *et al.*, 1993) and by being expressed in angioblasts of the head mesenchyme and in the veins of embryos, and selectively in lymphatic endothelia thereafter (Kaipainen *et al.*, 1995). The paracrine expression patterns of VEGF-C and VEGFR-3 in many tissues suggest that VEGF-C may function in angiogenesis of the lymphatic vasculature (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). On the other hand, the ability of VEGF-C to activate VEGFR-2 points to its possible functional redundancy with VEGF.

The VEGF-C precursor is more than twice as large as the mature polypeptide, initially isolated from PC-3 cell culture media (Joukov *et al.*, 1996). This, combined with the unusual structure of the precursor, raised questions

about the role of its proteolytic processing, possibly affecting receptor specificity, affinity and biological activity. These questions have been addressed in the present study.

Results

Characterization of VEGF-C antibodies and mapping of peptide epitopes in reduced and alkylated VEGF-C polypeptides

To study VEGF-C processing, we first generated antisera recognizing two different regions of the VEGF-C precursor. Antiserum 882 was obtained by immunization with a synthetic peptide corresponding to amino acid residues 2–18 of the N-terminus of mature secreted human VEGF-C [residues 104–120 of the VEGF-C prepropeptide (Joukov *et al.*, 1996); EMBL, GenBank and DDBJ entry X94216]. Antiserum 905 was raised against the N-terminus of the putative VEGF-C propeptide (residues 33–54) (see Figure 3). These antisera and the extracellular domain of VEGFR-3 (R-3EC) were then compared for their ability to bind metabolically labelled recombinant VEGF-C from the conditioned media (CM) of transfected 293-EBNA cells. Both antibodies precipitated VEGF-C forms with molecular masses of 15, 21, as well as a doublet of 29/31 kDa (Figure 1A, lanes 3 and 5, arrows). At higher levels of VEGF-C expression, polypeptides of 43 and 58 kDa were also detected in the immunoprecipitates (Figures 1B and 2). Importantly, both antibodies immunoprecipitated the VEGF-C forms which were able to bind VEGFR-3 (Figure 1A, lane 2). The doublet of 29/31 kDa was the major component of the immunoprecipitates. The 21 kDa band was precipitated by antiserum 905 less efficiently than by antiserum 882, suggesting that a fraction of this form is bound to (a) polypeptide(s) containing also the N-terminal VEGF-C sequence recognized by antiserum 905. Pre-treatment of the antisera with the corresponding peptides used for immunizations abolished their ability to immunoprecipitate the above-mentioned polypeptides (Figure 1A, lanes 4 and 6), indicating that they were specific for VEGF-C.

In order to explore the structure of the VEGF-C peptides further, we compared the abilities of the antisera to bind VEGF-C after reduction and alkylation of disulfide bonds. This treatment prevented the precipitation of the 29 and 43 kDa polypeptides by both antisera and of the 21 kDa form by antiserum 905 (Figure 1B, lanes 1–4). Reduction and alkylation slowed down the migration of the VEGF-C polypeptides in SDS–PAGE, presumably by dissociating intrachain bonds. Therefore, the absence of the 29 kDa form in these conditions could have been due to its co-migration with the 31 kDa component of the doublet. To show that this is not the case, we generated an artificial *N*-glycosylation site in the N-terminal part of VEGF-C by replacing Arg102 with a serine residue, resulting in the NSS(102) peptide (see Figure 3). This mutation slowed down the mobility of the polypeptide normally migrating at 31 kDa and therefore improved the separation of the doublet, thus confirming the above conclusion (data not shown). The mobilities of the 58 and 15 kDa forms were also reduced to 64 and 21 kDa respectively, indicating that these VEGF-C polypeptides contained the appropriate N-terminal peptide of VEGF-C (data not shown). On the other hand, the 21, 29 and 43 kDa forms were not affected by the R102S mutation, suggesting that these polypeptides contain peptide sequences located C-terminally of R102. The specificity of antiserum 905 was demonstrated further by its inability to immunoprecipitate a VEGF-C mutant in which the N-terminal propeptide (residues 32–102) was deleted (ΔN, see Figures 1B and 3). The ΔN polypeptide, immunoprecipitated with the 882 antiserum, migrated in SDS–PAGE with a mobility corresponding to the size of the deletion (~8 kDa) and it was co-precipitated with an equal amount of another pair of polypeptides of 29–32 kDa, which were not recognized by antiserum 882 upon reduction/alkylation of disulfide bonds. These polypeptides were considered to represent heterogenously cleaved/glycosylated C-terminal fragments of the ΔN precursor.

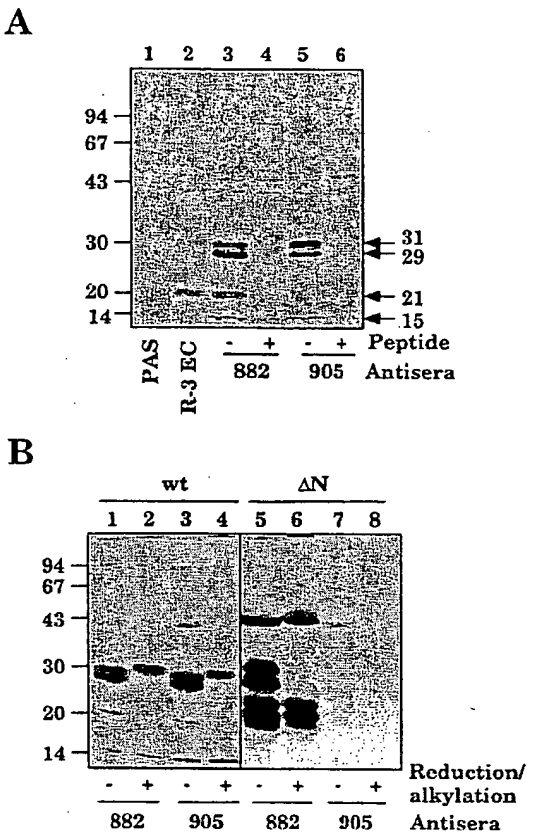


Fig. 1. Recognition of VEGF-C polypeptides by antibodies and VEGFR-3. 293-EBNA cells were transfected with VEGF-C, metabolically labelled, and secreted polypeptides were isolated from the medium with subsequent analysis by SDS–PAGE and autoradiography. (**A**) Wild-type VEGF-C was precipitated from CM using protein A–Sepharose (PAS) only (lane 1), PAS and R-3EC (lane 2), antiserum 882 (lanes 3 and 4) or antiserum 905 (lanes 5 and 6). Lanes 4 and 6 show immunoprecipitation using the antisera pre-treated with the corresponding peptides used for immunizations. R-3EC means recombinant soluble extracellular domain of VEGFR-3. (**B**) The antisera 882 and 905 were used to immunoprecipitate wt (lanes 1–4) or ΔN VEGF-C (lanes 5–8) from non-treated CM (lanes 1, 3, 5 and 7) or from CM treated with dithiothreitol and iodoacetamide to reduce and alkylate disulfide bonds (lanes 2, 4, 6 and 8).

Biosynthesis, dimerization and proteolytic processing of VEGF-C

To analyse the kinetics of VEGF-C biosynthesis and processing, we performed metabolic pulse–chase labelling

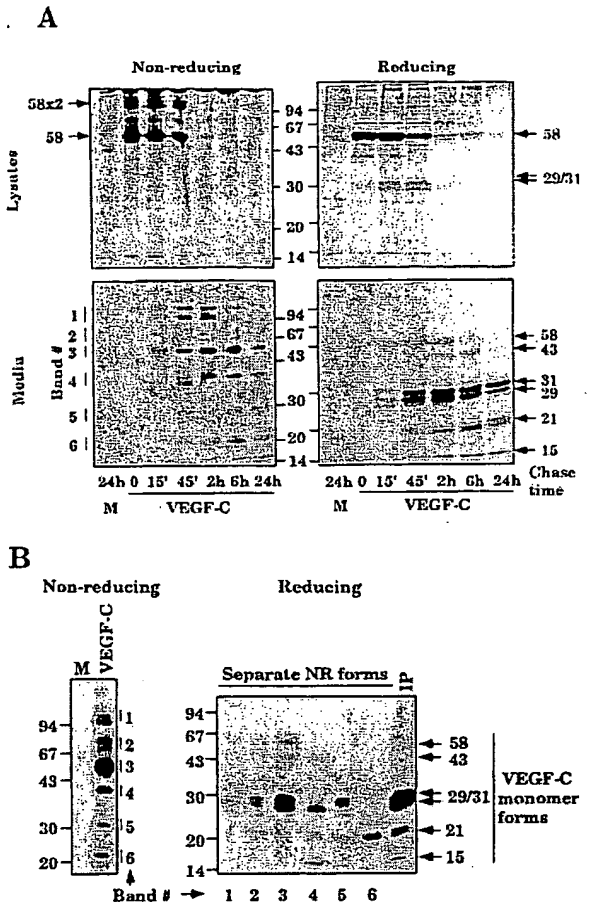


Fig. 2. Biosynthesis, dimerization and proteolytic processing of VEGF-C polypeptides. (A) Cells were metabolically labelled for 30 min and then chased in non-radioactive medium for the indicated periods of time. The media and cell lysates were immunoprecipitated with antiserum 882 and analysed by SDS–PAGE in 15% gel in non-reducing and reducing conditions. Mock-transfected cultures (M) were analysed after a 24 h chase period. Numbers on the right and on the upper left panels indicate molecular masses (kDa) of the VEGF-C forms. Band numbers of the lower left panel correspond to those in (B). (B) Labelled wt VEGF-C polypeptides were first separated in a non-reducing gel (left panel), excised and subjected to SDS–PAGE in reducing conditions (right panel). The corresponding band and lane numbers are indicated.

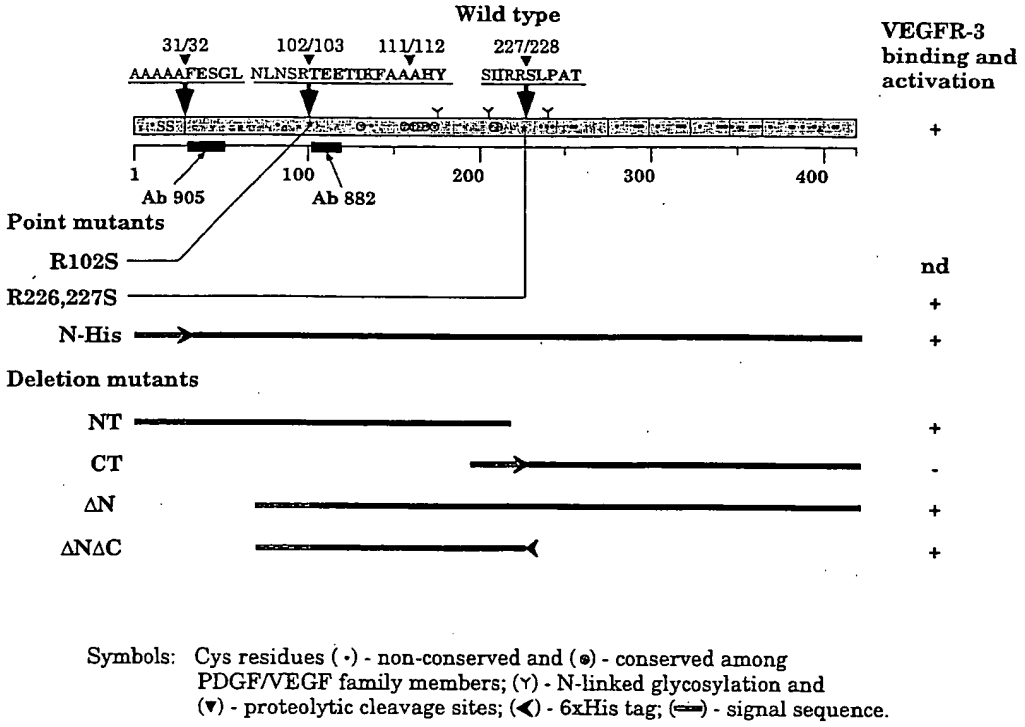
experiments with cells expressing recombinant VEGF-C. Analysis of the immunoprecipitated VEGF-C polypeptides after different chase periods in non-reducing and reducing conditions revealed that VEGF-C is first synthesized as a 58 kDa precursor, most of which undergoes dimerization before secretion into the culture medium (Figure 2A, upper panels, arrows '58' and '58×2' in lanes 0–45'). It is cleaved further, forming a 29 and a 31 kDa polypeptide (lanes 0–2 h, arrows 29/31), and rapidly secreted, as only a trace amount of the labelled protein was found intracellularly after a 2–6 h chase period. Most of the secreted VEGF-C was made of disulfide-linked low molecular weight forms at all time points analysed (Figure 2A, lower panels), indicating that proteolytic processing accompanies the secretion of VEGF-C. Proteolytic cleavage was detected in cell lysates at 0 min and in the media after a 15 min chase period, but the resulting chains of 31 and 29 kDa were held together by disulfide bonding (compare lanes 15'–2 h run in reducing and non-reducing conditions). At later chase times, these complexes were cleaved further, with concominant accumulation of a 15 and a 21 kDa polypeptide in reducing conditions (lanes 2–24 h). Importantly, this step of the processing occurs after secretion, as no 15 or 21 kDa forms were detected in the cell lysates (upper panels).

To analyse the composition of the different secreted VEGF-C forms we separated ^{35}S-labelled recombinant VEGF-C polypeptides by SDS–PAGE in non-reducing conditions, excised the polypeptide bands from the gel, reduced the disulfide bonds by treatment of the gel pieces with β-mercaptoethanol and re-analysed the polypeptides in reducing conditions (Figure 2B). The major part of the high molecular weight VEGF-C forms (bands 1–3) gave rise to 29/31 kDa doublets, confirming that the cleaved VEGF-C polypeptides are disulfide-bonded. Only a small fraction of the precursor protein is non-processed or partially processed (products of 58 and 43 kDa in the right hand panel). The low molecular weight components (lanes 4 and 5) contain heterodimerized 15, 21, 29 and 31 kDa polypeptides as well as homodimers of the 31 kDa polypeptide. Interestingly, the monomeric 21 kDa form was also detected (lane 6). The 15 kDa product was disulfide bonded only with the 29 kDa polypeptide (lane 4).

Identification of the proteolytically processed and disulfide-linked forms of VEGF-C

We next used the purified IgG fraction of antiserum 882 to isolate recombinant VEGF-C by affinity chromatography as described in Materials and methods. The purified material contained major polypeptides of 15, 21, 29–30 and 31–32 kDa (data not shown). These polypeptides were subjected to N-terminal amino acid sequence analysis, which gave the sequence NH_2-F(32)ESGLDLSDA-COOH for the 15 and 31–32 kDa polypeptides and the sequence NH_2-A(112)HYNTEILKS-COOH for the 21 kDa form. Because of our inability to obtain an N-terminal sequence for the 29–30 kDa polypeptide, we generated a VEGF-C construct, containing an N-terminal 6×His tag after the signal sequence (see Figure 3, N-His). Polypeptide components of 32 and 29 kDa were obtained after expression and affinity purification of N-His; analysis of the latter polypeptide revealed the N-terminal amino acid sequence NH_2-S(228)LPATL-COOH.

Comparison of the obtained sequences with the sequence of the VEGF-C precursor indicated that polypeptides of 15 and 31 kDa correspond to the N-terminal region of the secreted VEGF-C after cleavage of the signal peptide between Ala31 and Phe32 (Figure 3, arrowhead on the left). The 29 kDa form then represents the C-terminal half of the VEGF-C precursor generated by cleavage between Arg227 and Ser228 (arrowhead on the right). This polypeptide contains one putative *N*-linked glycosylation site and may be cleaved additionally at its C-terminus, as we could not isolate VEGF-C either by using an antiserum against the C-terminal amino acid residues 372–394 or by using the 6×His tag at its C-terminus (data not shown). The 21 kDa form is generated by cleavage of the VEGF-C precursor between Ala111 and Ala112 (grey arrowhead). This cleavage of the recombinant protein thus occurs nine residues C-terminal of the cleavage site located between Arg102 and Thr103, originally described in cultures of



Symbols: Cys residues (•) - non-conserved and (⊛) - conserved among PDGF/VEGF family members; (Y) - N-linked glycosylation and (▼) - proteolytic cleavage sites; (◀) - 6xHis tag; (═) - signal sequence.

Fig. 3. Schematic structure of the wild-type and mutant VEGF-C forms. The VEGF-C prepropeptide is depicted on the top. The signal sequence and the BR3P motifs are outlined. Peptide sequences adjacent to the proteolytic cleavage sites are also shown. Cleavage sites are indicated by arrows, arrowheads and by numbers of the flanking amino acid residues. Peptide sequences revealed by the N-terminal sequence analysis are marked in bold. The diagram under the box shows the scale in amino acid residues. The epitopes recognized by the antibodies 882 and 905 are marked in the diagram as black boxes. The sites of the point mutations are indicated by asterisks. Other VEGF-C mutants are shown as thick black lines, with the signal sequence marked in grey. The ability of the corresponding construct to bind and to activate VEGFR-3 is indicated on the right (nd, not determined).

PC-3 cells (Joukov *et al.*, 1996). Mature polypeptides of 21 and 31 kDa thus contain the entire VEGF homology domain with all eight conserved cysteine residues and two putative *N*-glycosylation sites.

Taking into account these results and analysis of VEGF-C composition in reducing and non-reducing conditions, one can conclude that the main fraction of processed VEGF-C consists of disulfide-bonded N- and C-terminal parts of VEGF-C precursor cleaved between Arg227 and Ser228 (bands 1–3 in Figure 2A and B). Migration of the proteins in non-reducing conditions suggests that the proteolytic processing occurs gradually. Band 1 presumably contains tetrameric complexes made of two 29/31 kDa dimers linked by disulfide bonds (Figure 2B). Analysis of band 2 suggests that it contains trimers made of the 29/31 kDa dimer disulfide-bonded with the 21 kDa form. It also includes small amounts of 43 and 58 kDa polypeptides. However, the major fraction of the 29 and 31 kDa polypeptides migrates in SDS–PAGE as a disulfide-bonded heterodimer (band 3, compare with bands 1 and 2), while most of the 21 kDa form migrates as a monomer (band 6). Some non-processed monomeric 58 kDa precursor and partially processed 43 kDa polypeptide are also included in band 3. Band 4 is formed mainly by the C-terminal half of the VEGF-C precursor (29 kDa), linked by disulfide bonds with its N-terminal fragment of 15 kDa, and band 5 contains the monomeric N-terminal half of the precursor and a small fraction of the 15 and 21 kDa forms heterodimerized by disulfide bonds. An identical processing pattern was observed when R102S VEGF-C was analysed to improve separation of the 29 and 31 kDa components of the doublet (data not shown).

VEGF-C is processed similarly in different cell types

To exclude the possibility that the observed VEGF-C processing pattern is cell type specific and/or occurs only in cells expressing extremely high VEGF-C levels, we analysed VEGF-C isolated from different transfected and non-transfected cells using the 882 antiserum. The main form of both endogenous VEGF-C, produced by PC-3 cells or HT1080 cells, and of the recombinant VEGF-C expressed in 293-EBNA, COS-7 and HT1080 cells is a doublet of 29/31 kDa. The 15, 21 and 58 kDa VEGF-C forms produced by PC-3 and 293-EBNA cells also had similar mobilities in SDS–PAGE. The proteolytic processing of the VEGF-C precursor in COS cells was less efficient when compared with other cell types, possibly due to a high level of expression or a species difference (data not shown). Taken together, these results indicate that VEGF-C is processed similarly in different cell types.

We further analysed whether the 21 kDa VEGF-C form could be produced by proteolytic cleavage of the 31 kDa form. Serum-free CM was collected from PC-3 cell cultures after various periods, concentrated and analysed by Western blotting using the antiserum 882. As can be seen from Figure 4A, the 21 kDa form accumulated in the medium during cell culturing. A similar product could not be detected in 293-EBNA cells, because cleavage of VEGF-C in these cells occurs more C-terminally (see

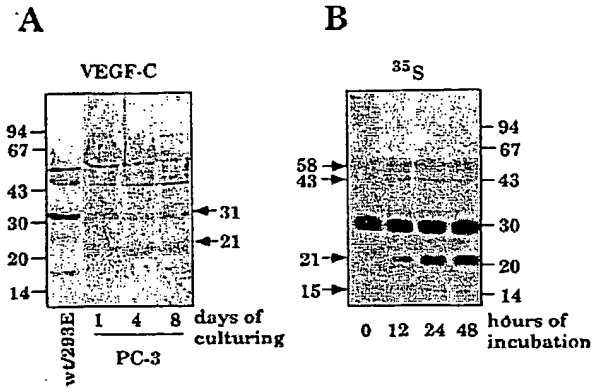


Fig. 4. Proteolytic processing of VEGF-C by secreted protease(s). (A) PC-3 cells were cultured for the indicated periods in FCS-free medium, which was then concentrated and analysed by Western blotting and detection using the 882 antiserum. Note that the mature VEGF-C 21 kDa form is detected in PC-3 cells, but not in 293-EBNA cells, transfected with VEGF-C (wt/293E). (B) CM from 293-EBNA cells, containing labelled VEGF-C, was incubated with concentrated CM from PC-3 cells for the indicated periods of time, and subjected to immunoprecipitation with antiserum 882. The precipitated material was analysed by SDS–PAGE and autoradiography. Note the accumulation of the mature 21 kDa form during incubation.

Figure 3) and thus deletes about half of the peptide sequence recognized by the antiserum. However, the remaining epitope appears to be sufficient for immunoprecipitation of the 21 kDa form using the same antiserum (Figures 1–3 and 4B). Addition of concentrated CM from PC-3 cells to medium containing ^{35}S-labelled recombinant VEGF-C also caused its proteolytic cleavage, with accumulation of the 21, 43 and 15 kDa products (Figure 4B, arrows), indicating that the protease responsible for VEGF-C cleavage is secreted to the medium. We also observed proteolytic cleavage of the recombinant 31 kDa VEGF-C polypeptide accompanied by simultaneous accumulation of the 21 kDa form upon long-term storage of the CM from transfected 293-EBNA cells (data not shown).

'Recombinantly processed' VEGF-C binds VEGFR-3 and VEGFR-2 with high affinity and induces receptor autophosphorylation

In order to identify and analyse biologically active VEGF-C polypeptides, we generated a panel of deletion mutants of VEGF-C based on the proteolytic processing sites (Figure 3). We found that the ability to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 depends on the presence of the VEGF homology domain. This conclusion is based on the activating properties of polypeptides encoded by the constructs VEGF-C wt, N-His, NT, ΔN, and ΔNΔC, schematically presented in Figure 3 (data not shown). The construct CT, in which the signal sequence was fused to Ser228 of the C-terminal cleavage site, was expressed efficiently and secreted to the culture medium, but it did not stimulate tyrosine phosphorylation of VEGFR-2 or VEGFR-3 (data not shown). The maximal receptor-stimulating activity corresponded to the 21 kDa form, in which both the N- and C-terminal propeptides were deleted at the proteolytic processing sites or in their close proximity (construct ΔNΔC) (see below).

We next produced the ΔNΔC protein in the *Pichia pastoris* yeast expression system and analysed its ability to bind to and stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2 expressed in porcine aortic endothelial (PAE) cells (Pajusola *et al.*, 1994; Waltenberger *et al.*, 1994). Non-transfected PAE cells did not show significant binding of radioiodinated purified ΔNΔC, while specific high affinity binding sites were detected in PAE/VEGFR-3 and PAE/VEGFR-2 cells (Figure 5). The affinities were 135 and 410 pM, respectively, based on Scatchard analysis of the binding data (Figure 5A and B). VEGF-C and VEGF competed with each other for VEGFR-2 binding, VEGF being more efficient in this respect, indicating that the binding involves overlapping sites of the receptor (Figure 5C and D). ΔNΔC, like VEGF, could also be cross-linked to VEGFR-2 on PAE cells (Figure 5F) and it bound to soluble extracellular domains of VEGFR-2 and VEGFR-3. This binding was eliminated by addition of a 30-fold excess of the non-labelled recombinant factor (data not shown). However, ΔNΔC bound neither to the VEGFR-1 extracellular domain (data not shown), nor to the VEGFR-1 expressed in PAE cells (Figure 5E).

Recombinant ΔNΔC, produced both by mammalian and yeast cells, stimulated tyrosine phosphorylation of VEGFR-3 and VEGFR-2 in a dose-dependent fashion at concentrations of 0.2–20 nM (Figure 5G and H and data not shown). This effect was not affected by the presence of the 6×His tag (data not shown). The stimulation of VEGFR-2 was comparable with that of similar concentrations of VEGF. Heparin at 1 μg/ml either did not affect or even decreased binding of ΔNΔC by both receptors (data not shown). Altogether, these data indicate that the proteolytically processed 21 kDa VEGF-C is a ligand and an activator of both VEGFR-3 and VEGFR-2.

Mature VEGF-C has VEGF-like activities

The ability of ΔNΔC to activate VEGFR-2 raised the question of whether it can also induce biological responses characteristic of VEGF. We found that ΔNΔC stimulated the proliferation of bovine capillary endothelial (BCE) cells, although equal stimulation required ~50-fold higher concentrations of VEGF-C in comparison with VEGF (Figure 6A). ΔNΔC, like wt VEGF-C, stimulated the migration of BCE cells in collagen gel, again at higher concentrations when compared with VEGF (Figure 6B). Also, pure recombinant ΔVEGF-C injected subcutaneously into guinea pig skin increased the permeability of blood vessels in a dose-dependent manner (Figure 7A). In this assay, only 4- to 5-fold higher concentrations of ΔNΔC were required compared with VEGF (Figure 7B). Altogether, these data indicate that the proteolytic processing of the VEGF-C precursor generates a biologically active factor which possesses VEGF-like effects on endothelial cells, stimulating their proliferation and migration, as well as the permeability of blood vessels *in vivo*.

Proteolytic maturation affects receptor specificity and activity of VEGF-C

We next addressed the question of whether proteolytic processing affects the ability of VEGF-C to bind and to activate VEGFR-3 and VEGFR-2. In addition to the above-described ΔNΔC, we also generated the VEGF-C R226,227S form in which Arg226 and Arg227, adjacent to the cleavage site, were replaced with serine residues

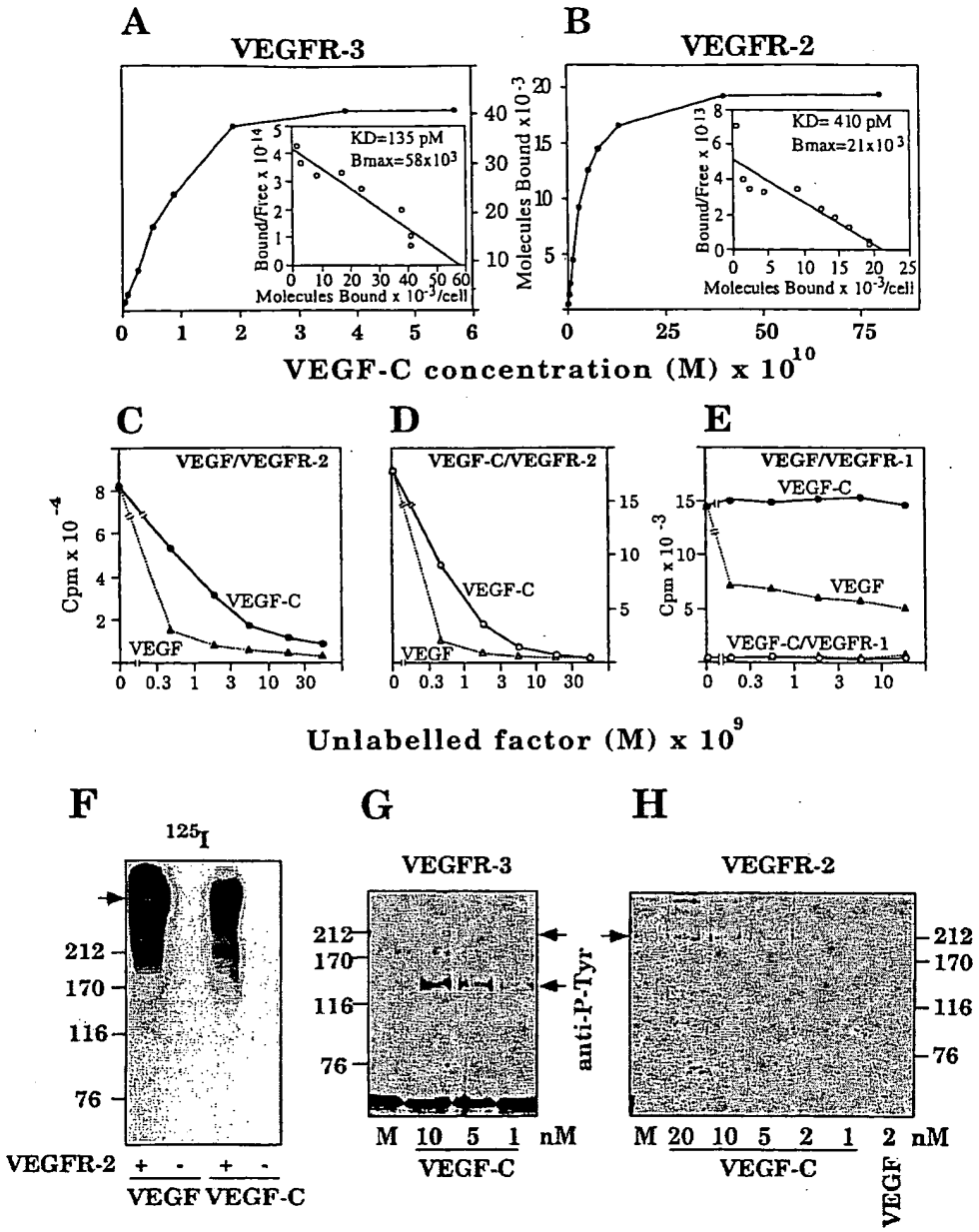


Fig. 5. VEGF-C binds to and activates VEGFR-3 and VEGFR-2. (**A**) and (**B**) Saturation binding curves and Scatchard analysis (inserts) of [^{125}I]ΔNΔC binding to PAE/VEGFR-3 cells (A) and PAE/VEGFR-2 cells (B). (**C**) and (**D**) Displacement of [^{125}I]VEGF (C) or [^{125}I]ΔNΔC (D) from VEGFR-2 by VEGF (triangles) and VEGF-C (circles). (**E**) Displacement of [^{125}I]VEGF (closed symbols) and [^{125}I]ΔNΔC (open symbols) from PAE/VEGFR-1 cells by VEGF (triangles) and ΔNΔC (circles). (**F**) Autoradiogram of VEGFR-2 immunoprecipitates from PAE (–) and PAE/VEGFR-2 (+) cells after cross-linking with [^{125}I]VEGF or [^{125}I]ΔNΔC. The arrow shows the mobility of the major labelled ligand–receptor complex. (**G**) and (**H**) Stimulation of tyrosine phosphorylation of VEGFR-3 and VEGFR-2 by ΔNΔC at different concentrations. Control lanes show analysis of mock-stimulated cells and treatment with 2 nM VEGF. The tyrosine-phosphorylated receptors are marked by arrows. Note the concentration-dependent phosphorylation of VEGFR-2, and of unprocessed 195 kDa and proteolytically processed 125 kDa VEGFR-3 forms in cells treated with ΔNΔC.

(Figure 3). As a consequence, the proteolytic processing at this site was almost completely abolished, as detected by Western blotting using the 882 antiserum (Figure 8A, lane 2). Small amounts of the 31 and 21 kDa polypeptides were, however, found in [^{35}S]R226,227S immunoprecipitates, possibly due to clevage at an alternative site (Figure 9B, lane 1). R226,227S can thus be considered an analogue of the VEGF-C precursor, while wt VEGF-C consists mostly of partially processed 29 and 31 kDa forms, ΔNΔC being an analogue of fully processed, mature VEGF-C (Figure 8A, lanes 3 and 4).

As can be seen from Figure 8B, all processed VEGF-C forms bind to R-3EC, with preferential binding of the 21 kDa form (lanes 2, 5, 7 and 13). Even more striking was the selective binding of the mature 21 kDa form of wt VEGF-C and of ΔNΔC by the VEGFR-2 extracellular domain–alkaline phosphatase fusion protein (R-2EC, lanes 3, 6, 8 and 14). Neither protein A–Sepharose (see Figure

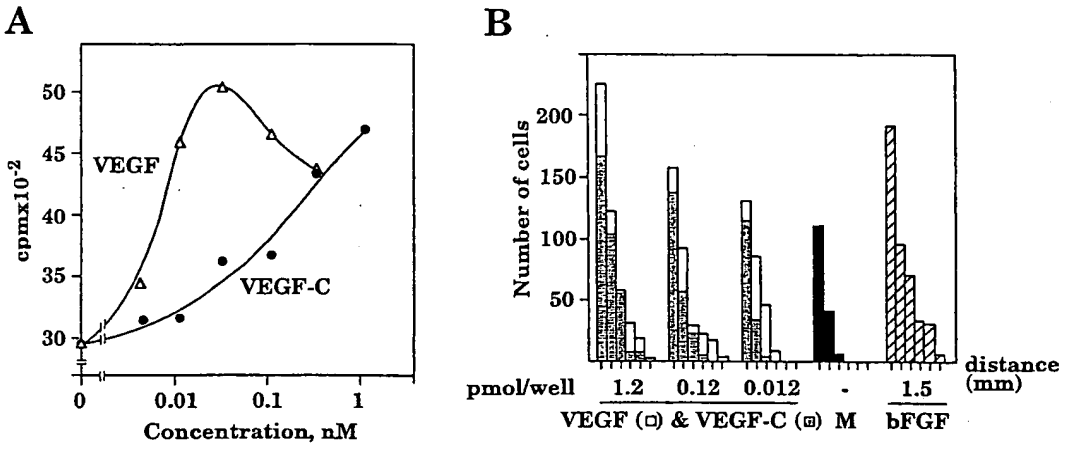


Fig. 6. VEGF-like activity of VEGF-C. (A) ΔNΔC stimulation of [^{3}H]thymidine incorporation into DNA of BCE cells. Results of one experiment using different concentrations of the factors are shown. Standard deviations were <10%. (B) Migration of BCE cells in collagen gel. The diagram shows the number of cells migrating to six different distances (0.6 μm step) starting from the left (marked by vertical ticks). Analysis employed mock medium (M, black bars) or medium with the indicated amounts of VEGF (open bars), ΔNΔC (grey bars) or bFGF (striped bars).

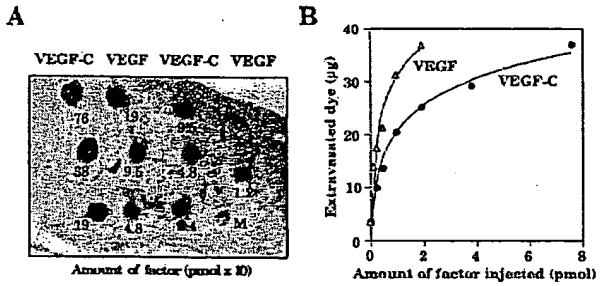


Fig. 7. Comparison of VEGF and VEGF-C in the Miles assay for vascular permeability *in vivo*. (A) The indicated amounts of VEGF and VEGF-C were injected intradermally to the back region of a guinea pig. The photograph shown was taken 20 min after the injections. Injection of diluent (PBS/BSA) is marked as M. (B) Graphs showing the quantitation of the results of the Miles assay as described in Materials and methods.

1, lane 1) nor protein G–Sepharose alone, or in combination with the anti-alkaline phosphatase antibodies (lanes 10 and 11), bound the 21 kDa form, although very small amounts of the the 58 and 31 kDa VEGF-C forms were bound unspecifically (lanes 8, 9 and data not shown). The specificity of VEGF-C binding was supported by the finding that R-2EC bound VEGF, but not PlGF, while R-3EC did not bind VEGF (data not shown).

Analysis of the receptor-bound material in non-reducing conditions revealed that the 60 kDa polypeptide, which was bound preferentially to R-3EC consisted of disulfide-bonded 29 and 31 kDa heterodimers (lane 13, upper arrow). Most of the 21 kDa polypeptide bound to both R-3EC and R-2EC migrated as a monomer in these conditions (lanes 13 and 14, lower arrow). This finding was most surprising with regard to previously published data concerning other VEGF family members (Maglione *et al.*, 1991; Heldin *et al.*, 1993; Olofsson *et al.*, 1996a; Ferrara, 1997).

We next analysed the ability of the described VEGF-C forms to compete with [^{125}I]VEGF-C(ΔNΔC) for binding to VEGFR-2 and VEGFR-3. As can be seen from Figure 8C, all VEGF-C mutants displaced [^{125}I]VEGF-C from VEGFR-3. The efficiency of displacement was as follows: ΔNΔC>wt>R226,227S, i.e. enhanced binding was obtained upon inclusion of the more mature forms. Recombinant VEGF165 failed to displace VEGF-C from VEGFR-3, but VEGF, ΔNΔC and wt VEGF-C efficiently displaced labelled VEGF-C from VEGFR-2, ΔNΔC being more potent in comparison with wt VEGF-C (Figure 8D). The non-processed R226,227S form showed only weak competition with [^{125}I]VEGF-CΔNΔC, which could be attributed either to its much lower affinity for VEGFR-2, or to the presence of a small amount of the mature forms, cleaved at an alternative site (see above).

Next, we studied the ability of the above-mentioned VEGF-C forms to stimulate tyrosine phosphorylation of VEGFR-3 and VEGFR-2. Stimulation of VEGFR-3 and VEGFR-2 autophosphorylation by the different VEGF-C forms in general correlated with their binding properties and with the degree of proteolytic processing (Figure 8E). ΔNΔC showed a higher activity than wt VEGF-C (lanes 3 and 4), and R226,227S had a considerably weaker effect on autophosphorylation of VEGFR-3, and almost no effect on VEGFR-2 autophosphorylation (lane 2). Finally, the ability of different VEGF-C forms to promote vascular permeability was examined in the Miles assay. CM containing the VEGF-C polypeptides were pre-treated with monoclonal anti-VEGF neutralizing antibodies to eliminate the effect of endogenous VEGF produced by 293-EBNA cells. Although the effect of pure VEGF was neutralized in control experiments, the antibody-treated CM still slightly increased vascular permeability, presumably due to the presence of other permeability factors (Figure 8F and data not shown). CM containing wt and ΔNΔC VEGF-C increased vascular permeability, while the effect of R226,227S CM did not differ significantly from that of CM from mock-transfected cells (Figure 8F). Importantly, identical dilutions of CM were used for these experiments and for the experiments presented in Figure 8C–E. A Western blot analysis of CM using anti-VEGF-C antiserum 882 is shown in Figure 8A to illustrate the relative amounts of the factors present.

Because antiserum 882 did not recognize the mature wild-type polypeptide produced by 293-EBNA cells on a Western blot (see above), metabolic labelling and immuno-

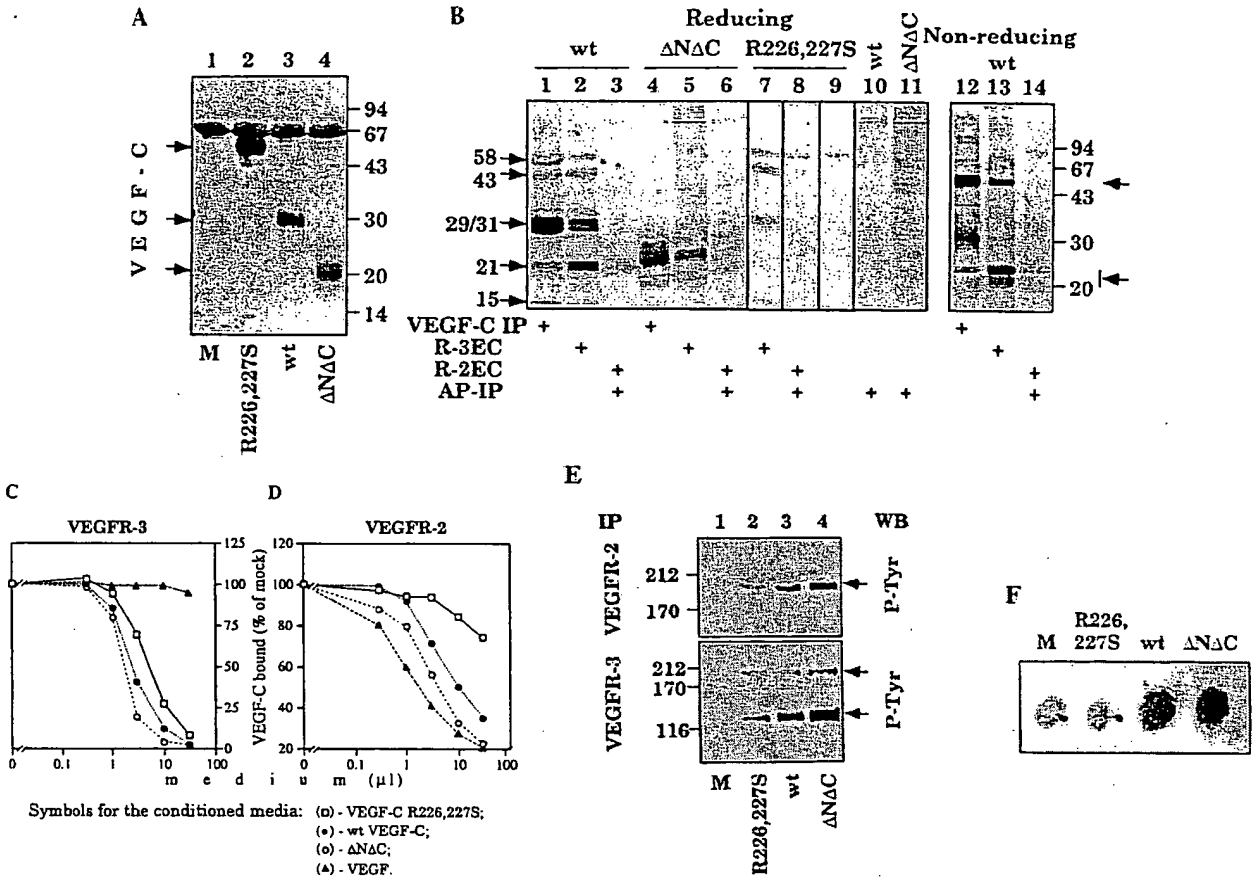


Fig. 8. Proteolytic processing increases the ability of VEGF-C to bind to and activate VEGFR-3 and VEGFR-2. (**A**) Detection of three processed VEGF-C forms by antiserum 882 on a Western blot (arrows). VEGF-C concentrations in CM were adjusted by dilutions with medium from mock-transfected cells. The band at 67 kDa is BSA added as a carrier protein. (**B**) Binding of the various VEGF-C forms by the extracellular domains of VEGFR-3 and VEGFR-2. Metabolically labelled VEGF-C from CM of cells transfected with the indicated constructs was bound to R-3EC (lanes 2, 5 and 7) or to R-2EC (lanes 3, 6 and 8). The receptor extracellular domains were precipitated and analysed by SDS–PAGE. The same amounts of CM were immunoprecipitated (lanes 1 and 4) or treated with PAS only (lane 9). Analysis of material in lanes 1–3 in non-reducing conditions is shown in lanes 12–14. Note that the main fraction of the 21 kDa form bound to the extracellular domains of both receptors does not form disulfide-linked dimers (lower arrow), while most of the 29/31 kDa complexes bound to R-3EC are heterodimerized via disulfide bonds (upper arrow). (**C**) and (**D**) Displacement of [^{125}I]ΔNΔC from the receptors by the VEGF-C forms. PAE/VEGFR-3 (C) and PAE/VEGFR-2 (D) cells were incubated with trace amounts of [^{125}I]ΔNΔC in the absence or presence of different amounts of CM containing the indicated polypeptides, the cells were washed and the amount of bound radioactivity was measured in a γ-counter. Experiments presented in (C), (D) and (E) were carried out using the CM analysed in (A). (**E**) Stimulation of tyrosine phosphorylation of VEGFR-2 (upper panel) and VEGFR-3 (lower panel) by CM from mock-transfected cells (lane 1) and from cells overexpressing the indicated VEGF-C forms. Tyrosine-phosphorylated receptor polypeptides are indicated by arrows. (**F**) Proteolytically processed VEGF-C increases vascular permeability. CM containing ~8 pM of the indicated VEGF-C variants were pre-treated with anti-VEGF neutralizing antibodies and injected intradermally to the back of a guinea pig. The photograph shown was taken 20 min after the injections.

precipitation was carried out to better estimate the relative amounts of each processed form (Figure 9B, lanes 1, 3 and 5). In the experiment presented in Figures 8 and 9, the amount of the 21 kDa polypeptide was approximately one-third of that of the 31 kDa form in the same wt VEGF-C conditioned medium. Taken together, these data indicate that the ability to bind to and to activate VEGFR-3 and VEGFR-2 increases during the proteolytic processing of VEGF-C. Non-processed VEGF-C preferentially binds to and activates VEGFR-3, while the mature 21 kDa VEGF-C form is a high affinity ligand and an activator of both VEGFR-3 and VEGFR-2.

Mature form of VEGF-C consists of non-covalent dimers

Members of the PDGF/VEGF family are active only as dimers. However, as shown above, the proteolytically processed VEGF-C exists mainly as a monomer or a non-disulfide-bonded dimer, which binds VEGFR-3 and VEGFR-2. We were interested in the possibility that dimerization of the processed VEGF-C occurs via non-covalent interactions. Unlike VEGF, which migrates in non-reducing conditions as a dimeric protein of ~44 kDa, most of ΔNΔC migrates as a monomer (Figure 9A). As can be seen from Figure 9B, lanes 6 and 8, about one-half of disuccinimidyl suberate (DSS)-cross-linked VEGF and ΔNΔC migrated as dimers (arrows pointing to lanes 6 and 8 on the right) in reducing conditions. Taking into account that in our conditions ~90% of VEGF migrated as a disulfide-bonded dimer (Figure 9A, lane 1), we conclude that mammalian cells produce ΔNΔC preferentially as a non-covalently bonded dimer (Figure 9A, lane 2 and Figure 9B, lanes 5 and 6). When wt VEGF-C was cross-linked, the amount of the 21 kDa form was

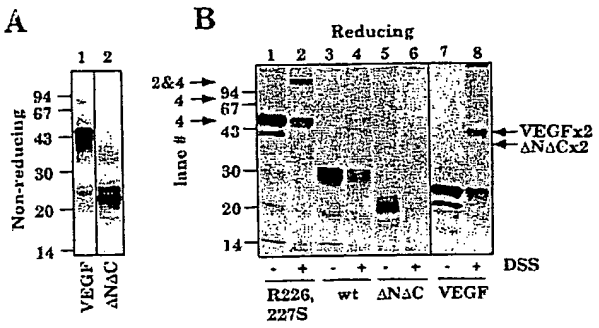


Fig. 9. Mature 21 kDa VEGF-C forms non-covalent dimers. (A) Metabolically labelled VEGF165 (lane 1) and ΔNΔC (lane 2) immunoprecipitated from CM and analysed by SDS–PAGE in non-reducing conditions. Note that the majority of VEGF migrates as a dimeric protein, while ΔNΔC migrates as a monomer in these conditions. (B) Analysis of the VEGF and VEGF-C forms in CM with (+) or without (–) covalent cross-linking using DSS, followed by immunoprecipitation with the antiserum 882 (lanes 1–6) or with VEGF antibodies (lanes 7 and 8). The numbers and arrows point to the cross-linked dimers and multimers detected in the corresponding lanes. Note that approximately equal proportions of ΔNΔC and VEGF migrate as dimers upon cross-linking (arrows on the right). Note also that complexes of ~ 60 and 80 kDa appear upon cross-linking of wt VEGF-C, and complexes of ~120 kDa are formed when R226,227S is cross-linked (arrows on the left).

considerably decreased (Figure 9B, lane 4), suggesting that it is bound to other polypeptides. Also, additional bands of 60, 80 and 120 kDa appeared in reducing conditions (lane 4, arrows on the left). The first of these apparently represents heterodimers of 29 and 31 kDa forms; the 80 kDa complex is most probably a trimer, consisting of 21, 29 and 31 kDa polypeptides, and the 120 kDa band contains two dimerized VEGF-C precursors, most of which are cleaved at the 227R/228S site. When cleavage between Arg227 and Ser228 was abolished (the R226,227S mutant), no cross-linked complexes of 60 and 80 kDa were detected; instead complexes of ~120 kDa were very prominent, both in non-reducing conditions (data not shown) and upon cross-linking (lane 2). These complexes presumably consist of non-processed VEGF-C dimers linked by disulfide bonds. Despite the fact that we were unable to cross-link the complexes completely, these data, along with the analysis of VEGF-C in reducing and non-reducing conditions, clearly show the co-existence of a variety of its di- and multimeric forms, assembled via disulfide bonding and non-covalent interactions. We also found that recombinant N- and C-termini of VEGF-C were able to form heterodimers when co-expressed in mammalian cells (data not shown), emphasizing the existence of a mechanism for the formation of such dimers in mammalian cells.

Discussion

Proteolytic processing of VEGF-C

Based on the described results, we propose the VEGF-C proteolytic processing model, which is presented schematically in Figure 10. This model resembles the model for the proteolytic processing of PDGF, especially of PDGF-BB (Östman *et al.*, 1988, 1992) in that: (i) the proteolytic cleavages occur after the formation of disulfide-bonded precursor dimers; (ii) both N- and C-terminal propeptides are subject to cleavage; and (iii) a variety of processed forms are secreted. On the other hand, there are several important differences between PDGF-BB and VEGF-C, concerning both their processing and the structure of the mature growth factors.

VEGF-C is released rapidly from cells upon secretion. Upon biosynthesis, two VEGF-C polypeptides, oriented in an anti-parellel fashion, form a dimer linked by disulfide bonds and apparently also by non-covalent bonds. Anti-parallel dimerization is supported by the disulfide bonding of the N- and C- terminal halves (29/31 kDa doublet) of the precursor. Precursor homodimerization is thus followed by the key event in the proteolytic processing—the cleavage between Arg227 and Ser228 dividing the VEGF-C precursor into nearly equal halves. This cleavage site was confirmed by N-terminal peptide sequence analysis and by the R226,227S substitutions, which abolished the cleavage. The N-terminal part of the 31 kDa form contains the VEGF homology domain, and the cysteine-rich C-terminus of the 29 kDa form contains the BR3 motifs. Similarly to PDGF, this processing step occurs in the producer cells, either close to the end of the secretory pathway or at the plasma membrane, because only small amounts of cleaved VEGF-C precursor can be detected in the cell lysates. Most of the secreted VEGF-C is then already cleaved between Arg227 and Ser228, and the resulting polypeptides initially form a tetramer, originating from two precursor polypeptides bound to each other. This processing step is probably conserved in evolution, because the human, mouse and avian VEGF-Cs, as well as FIGF, contain the same amino acid sequence, SIIRRS, surrounding the cleavage site, and a doublet of polypeptides of ~30 kDa is detected in the corresponding immunoprecipitates from the CM of transfected cells (Joukov *et al.*, 1996; Kukk *et al.*, 1996; Orlandini *et al.*, 1996). Morover, most of the VEGF-C secreted by different cell types migrates in reducing conditions as a doublet of 29/31 kDa (data not shown).

The efficient secretion of the R226,227S mutant as well as the presence of small amounts of unprocessed wt VEGF-C precursor in the CM indicate that the intracellular proteolytic cleavage is not a prerequisite for VEGF-C secretion. The C-terminal cleavage of the PDGF-BB precursor occurs in close proximity to the site which corresponds to Arg227 in VEGF-C. Cleavage of high molecular weight VEGF forms by plasmin, with release of diffusible VEGF, also takes place only 10 amino acid residues N-terminal of the VEGF-C cleavage site when VEGF and VEGF-C sequences are aligned (Houck *et al.*, 1992; Keyt *et al.*, 1996a). The proteases responsible for the cleavage of these growth factors might differ, however, because of differences in peptide sequences surrounding the cleavage sites (Östman *et al.*, 1988).

The next step of the proteolytic processing of VEGF-C, which removes the N-terminal propeptide, occurs extracellularly, because the 21 kDa polypeptide was not detected in cell lysates. This differs from the proteolytic processing of PDGF, which occurs exclusively intracellularly (Östman *et al.*, 1992). The 21 kDa form accumulated even upon incubation of cell-free CM, indicating that the cleavage is catalysed by (an) as yet unknown secreted protease(s). Differences in the cleavage sites in cultures of PC-3 and 293-EBNA cells (A111/A112 and R102/

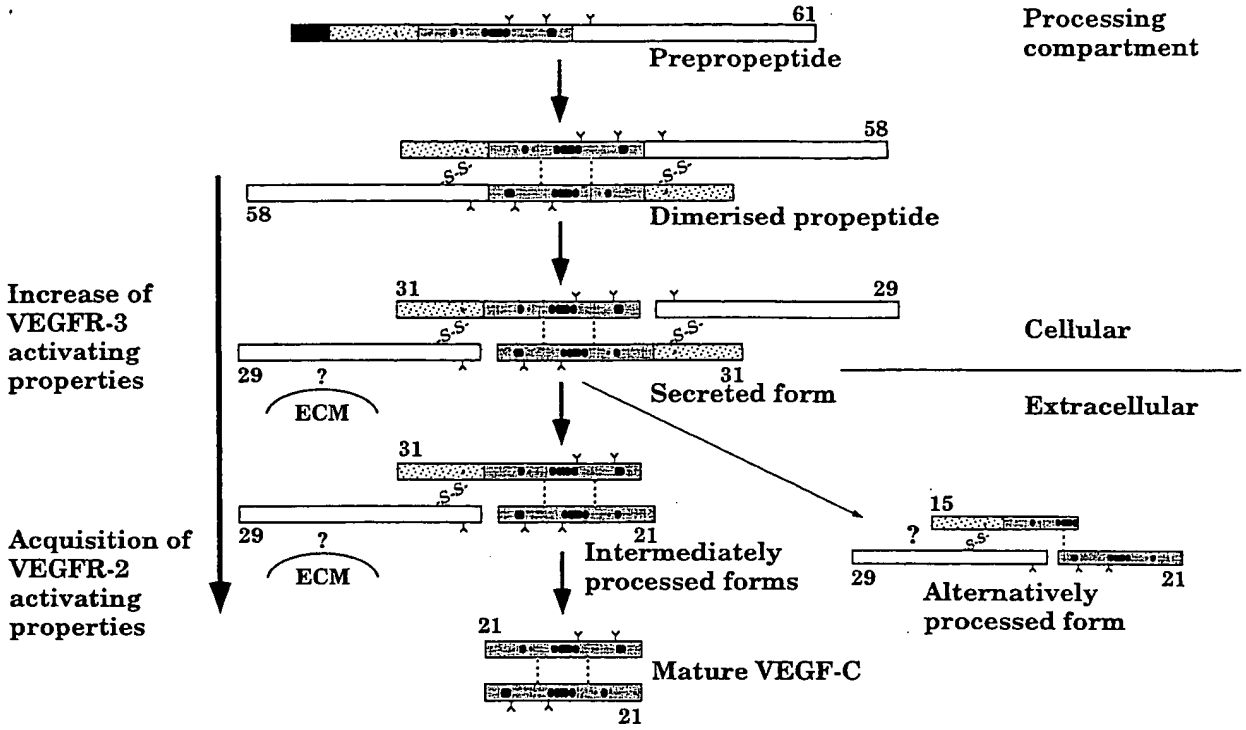


Fig. 10. Schematic model of the proteolytic processing of VEGF-C. The regions of VEGF-C polypeptide are marked as follows: signal sequence, black box; VEGF homology domain, grey box; N-terminal and C-terminal propeptides, dotted and open boxes, respectively. Cysteine residues and putative sites of N-linked glycosylation are shown as in Figure 3; the cysteine residues in the C-terminal propeptide are not marked for clarity. Numbers indicate molecular mass (kDa) of the corresponding polypeptide in reducing conditions. Disulfide bonds are marked as -S-S-; non-covalent bonds as dotted lanes. The hypothetical binding of the C-terminal domain to the extracellular matrix (ECM), and the proposed structure of the alternatively processed VEGF-C are indicated with question marks. The proteolytic generation of a small fraction of disulfide-linked 21 kDa forms is not indicated in the figure. Several intermediate forms are also omitted to simplify the scheme.

T103, respectively) suggest that such proteases are either redundant or that cell type-specific factors determine the exact cleavage sites. This stage of the proteolytic processing occurs in a more gradual fashion, and it finally gives rise to mature VEGF-C, composed of two VEGF homology domains bound by non-covalent interactions.

The small amounts of the shortest identified VEGF-C polypeptide of 15 kDa represent the N-terminal part of the precursor, which binds via disulfide bond(s) with the C-terminal 29 kDa propeptide. The mobility of the 15 kDa form in SDS–PAGE and its recognition by both antisera used in the present study suggest that it contains most of the VEGF homology domain, excluding the first glycosylation site, indicating the existence of an additional proteolytic processing site. Interestingly, this form is very similar to the short splicing variant (clone vh 1.1), reported for VRP (Lee *et al.*, 1996), and thus it may have an analogous, so far unknown function. It is possible that the 15 kDa polypeptide interacts with the 21 kDa form, giving rise to a trimer. It might have an antagonistic activity, competing with the mature ligand for receptors. Small amounts of a secreted 43 kDa form were also detected, but we could not isolate enough of this form to determine its peptide sequence. However, the inability of both VEGF-C antisera to precipitate this form upon reduction/alkylation of disulfide bonds and the correlation of its appearence with the appearance of the 15 kDa form suggest that it might represent the complementary C-terminal part of the VEGF-C precursor after cleavage of the 15 kDa N-terminal part.

Several lines of evidence indicate that mature VEGF-C made by transfected overexpressing cells is a non-covalent dimer. Most of the mature VEGF-C and ΔNΔC migrate at 21 kDa in reducing and non-reducing conditions. Despite this, similar proportions of dimeric molecules (~50%) are detected upon cross-linking the recombinantly produced VEGF and ΔNΔC. Of the various forms, these have the highest affinity for VEGFR-3 and VEGFR-2. The tetra- and trimeric VEGF-C molecules, which were detected upon cross-linking, presumably involve both disulfide bonds connecting the N- and C-terminal parts of separate precursor chains and non-covalent interactions between the two VEGF homology domains (see Figure 10). Subsequent removal of the N-terminal propeptide from both precursors would then explain the formation of the non-disulfide-linked mature VEGF-C dimer.

Absense of interchain disulfide bonds is unusual for the members of the PDGF/VEGF family, in which the second and fourth cysteine residues are involved in anti-parallel interchain disulfide bonds (Andersson *et al.*, 1992; Pötgens *et al.*, 1994). These disulfide bonds are crucial for dimerization and biological activity of VEGF, but are not required for dimerization or mitogenic effects of PDGF-BB (Andersson *et al.*, 1992; Kenney *et al.*, 1994; Pötgens *et al.*, 1994). It has also been shown that the dimer interface in PDGF-BB is sufficient to stabilize the dimer substantially in the absence of a covalent linkage (Oefner *et al.*, 1992). It is possible that such an interaction of the mature polypeptide chains is tighter in VEGF-C than in PDGF-BB. Interestingly, the mature VEGF-C contains an extra cysteine residue at position 137, located between

the first and the second cysteine residue characteristic of the PDGF/VEGF family. This residue is also conserved in mouse and avian VEGF-C (Kukk *et al.*, 1996; A.Eichmann *et al.*, unpublished data) and in FIGF (Orlandini *et al.*, 1996). This residue remains unpaired after cleavage of the N-terminal propeptide, which contains another unpaired cysteine residue.

Properties of the mature VEGF-C

The results with ΔNΔC, which mimics mature VEGF-C, support our earlier observations (Joukov *et al.*, 1996; Kukk *et al.*, 1996) and clearly indicate that proteolytically processed VEGF-C binds to and activates both VEGFR-3 and VEGFR-2. A single class of high affinity sites was observed in PAE/VEGFR-3 cells (K_D = 135 pM) and PAE/VEGFR-2 cells (K_D = 410 pM). These values are of similar magnitude to the affinities reported for the VEGF–VEGFR-2 interaction (Terman *et al.*, 1992; Waltenberger *et al.*, 1994). VEGF-C and VEGF displace each other from VEGFR-2, indicating that the same region of this receptor is involved in binding of both ligands. Surprisingly, none of the three basic residues reported to be critical for VEGFR-2 binding by VEGF (Keyt *et al.*, 1996b) are conserved in VEGF-C, indicating that other residues of VEGF-C are important for its interaction with VEGFR-2. VEGF-C also dose-dependently stimulated autophosphorylation of VEGFR-3 and VEGFR-2 but, in agreement with previously reported data (Lee *et al.*, 1996), we could not detect binding of VEGF-C to VEGFR-1.

Like VEGF, VEGF-C stimulates the proliferation and migration of endothelial cells and increases vascular permeability, albeit at concentrations higher than required for VEGF. These activities are probably mediated through VEGFR-2 activation (Park *et al.*, 1994; Waltenberger *et al.*, 1994). Higher effective concentrations of VEGF-C may depend on its lower affinity for VEGFR-2, and on its inability to bind VEGFR-1, precluding the formation of VEGFR-1–VEGFR-2 heterodimers, which may be required for maximal biological responses to VEGF (Waltenberger *et al.*, 1994; DiSalvo, 1995; Cao *et al.*, 1996; Clauss *et al.*, 1996). The role of VEGFR-2 in the effects of VEGF-C *in vivo* remains to be studied.

The paracrine relationship between the VEGF-C and VEGFR-3 expression patterns in embryos suggests that VEGF-C functions in the formation of the venous and lymphatic vascular systems, where VEGFR-3 is expressed (Kaipainen *et al.*, 1995; Kukk *et al.*, 1996). Our unpublished observations from transgenic mice support such a notion (Jeltsch *et al.*, 1997). However, the redundancy of VEGF-C with VEGF in VEGFR-2-mediated signalling might account for the interesting observations that VEGF –/– mice have delayed endothelial cell differentiation, while in VEGFR-2 –/– mice both haematopoietic and endothelial cell development is aborted, suggesting that (a) VEGFR-2 ligand(s) distinct from VEGF (such as VEGF-C) might play an important role in these processes (Shalaby *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Indeed, VEGF-C expression is first detected in day 7 p.c. embryos (Kukk *et al.*, 1996), which is striking, considering the first appearance of the VEGFR-3 mRNA on day 8.5 of gestation (Kaipainen *et al.*, 1995). The question of whether VEGF-C is indeed another factor essential for the development of haematopoietic/endothelial cells will need further studies.

Significance of the proteolytic processing of VEGF-C

Our results demonstrate that during proteolytic processing, VEGF-C acquires the ability to bind to and to activate VEGFR-2, and increases its affinity and activating properties towards VEGFR-3. Many other cytokines and growth factors are also synthesized initially as precursors. These include members of the epidermal growth factor (EGF) family, and the transforming growth β (TGF-β) superfamily, interleukins 1α and 1β, nerve growth factor, hepatocyte growth factor (HGF) and others. Proteolytic processing of TGF-β and HGF precursors is an essential step in the formation of the biologically active ligands (Naka *et al.*, 1992; Vigna *et al.*, 1994; Dubois *et al.*, 1995). Our data indicate that the proteolytic processing of VEGF-C plays a similar role, endowing the mature polypeptide with the ability to activate VEGFR-2. Taking into account the presence of VEGFR-2 in many types of endothelia and the broad expression pattern of VEGF-C, we propose that the biosynthesis of VEGF-C as a precursor prevents unnecessary angiogenic effects, elicited via VEGFR-2, and allows VEGF-C to signal preferentially via VEGFR-3, which is restricted to the venous endothelia during early stages of development and to the lymphatic endothelium during later stages. In certain circumstances, proteolytic processing would release mature VEGF-C, which is able to signal via both VEGFR-3 and VEGFR-2. It is also possible that activation of both VEGFR-3 and VEGFR-2, either as homo- or as heterodimers, is necessary to elicit a complete biological response to VEGF-C. In this case, proteolytic processing might provide a regulatory mechanism which gives the possibility of fine tuning the biological functions of VEGF-C. Also, the extracellular processing step introduces an additional level of regulation of the VEGF-C activity.

An important function of the proteolytic processing of PDGF, and possibly also of certain VEGF isoforms, is to control the bioavailability of the growth factor by removal of the C-terminal propeptide, containing a short stretch of positively charged amino acid residues responsible for the retention of the molecule at the cell surface or in the pericellular matrix (La Rochelle *et al.*, 1991; Houck *et al.*, 1992; Keyt *et al.*, 1996a). The resulting effect is similar to that of alternative splicing, which generates polypeptide variants devoid of the retention domain. VEGF-C is secreted readily into the CM, independently of whether it is processed or not. The isolated C-terminal half of VEGF-C is also released efficiently into the CM, when provided with the VEGF-C signal sequence. In addition, VEGF-C does not bind to heparin, which is known to interact with the basic regions of the long VEGF splice isoforms. The short stretch of basic amino acids, located at the C-terminus of the VEGF-C precursor (residues 372–386), either does not affect its secretion or is proteolytically removed. These data suggest that the bioavailability of VEGF-C is not regulated by the same mechanism as in the case of PDGF and VEGF. The propeptides also do not seem to be essential for the folding, assembly or secretion of VEGF-C homodimers, as the ΔNΔC form

was secreted efficiently as a dimer, and possessed all the tested activities of naturally processed VEGF-C.

The major secreted VEGF-C form contains the C-terminal propeptide which has an unusual structure with tandemly repeated cysteine-rich motifs and is linked via disulfide bonds to the N-terminal propeptide. The possible function of this, apparently by itself inactive C-terminal half of VEGF-C is unknown. Besides its striking similarity to the secretory silk protein (BR3P), the C-terminal VEGF-C propeptide also contains short motifs homologous to the EGF-like domains of other secreted proteins, most importantly of the extracellular matrix components such as fibrillin, laminin and tenascin. All of these proteins are known to participate in protein–protein or protein–cell surface interactions (Apella *et al.*, 1988). This observation, together with increasing evidence that the binding of growth factors to the extracellular matrix is a major mechanism regulating growth factor activity (Taipale and Keski-Oja, 1997), suggests that the secreted VEGF-C, which is proteolytically cleaved at the R227/S228 site, may stay associated with the extracellular matrix via its C-terminal propeptide (Figure 10). The unique organization of the BR3 motifs, which differ from previously known repeated motifs in secreted proteins of vertebrata, might provide additional specificity to the VEGF-C association with the extracellular matrix (Figure 10). Cleavage of the N-terminal propeptide would then release the active VEGF-C not only from the latent state, but also from its association with the extracellular matrix (Figure 10). In fact, some similarity can be seen between structural organization of secreted VEGF-C and TGF-β, with the N- and C- terminal VEGF-C propeptides being functional homologues of the TGF-β latency-associated protein and the latent TGF-β-binding protein respectively. The latter has a domain structure and is similar to fibrillin (reviewed in Miyazono *et al.*, 1994; Taipale and Keski-Oja, 1997). The questions of whether secreted VEGF-C indeed remains associated with the extracellular matrix and which protease is responsible for the proteolytic processing of VEGF-C remain to be answered in the future.

Materials and methods

Cell culture, transfections and metabolic labelling

293-EBNA cells, COS-7 cells, and HT1080 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)–10% fetal calf serum (FCS); PC-3 cells in Ham's F12 medium–7% FCS; PAE-KDR, PAE-Flt1 (Waltenberger *et al.*, 1994) and PAE-Flt4 (Pajusola *et al.*, 1994) cells in Ham's F12 medium–10% FCS. BCE cells (Folkman *et al.*, 1979) were cultured as described in Pertovaara *et al.* (1994). Cell transfections were carried out using the calcium phosphate precipitation method. An equivalent amount of the pREP7 plasmid without insert was used in mock transfections. When used for stimulation experiments, and for detection of VEGF-C expression by Western blotting, the culture medium was changed to DMEM–0.1% bovine serum albumin (BSA) 48 h after transfection and, after an additional 48 h, this medium was collected, clarified by centrufugation, concentrated using Centriprep-10 or Centricon-10 devices (Amicon) and used in the experiments. Metabolic labelling of non-transfected cells and cells transfected with VEGF-C constructs was carried out by addition of 200 and 100 μCi/ml respectively of Pro-mix™ L-[^{35}S] *in vitro* cell labelling mix (Amersham) to the culture medium devoid of cysteine and methionine. After 4 h, the medium was collected or, in some experiments, replaced with DMEM–0.1% BSA, and after an additional incubation for 4 h the media were combined, cleared by centrifugation and used for the immunoprecipitations.

Generation of VEGF-C antisera

Antisera 882 and 905 were generated by immunization of rabbits with synthetic peptides, corresponding to residues 104–120 (NH_2-EETIKFAAAHYNTEILK-COOH) and 33–54 (NH_2-ESGLDLSDAEPD-AGEATAYASK-COOH). The peptides were synthesized as a branched polylysine structure K3PA4 having four peptide acid chains attached to two available lysine residues. The synthesis was performed on a 433A Peptide Synthesizer (Applied Biosystems) using Fmoc-chemistry and TentaGel S MAP RAM10 resin mix (RAPP Polymere GmbH). Cleaved peptides were purified by reverse phase HPLC, dissolved in phosphate-buffered saline (PBS), mixed with Freund's adjuvant and used for immunizations of rabbits at bi-weekly intervals according to standard procedures. Antisera obtained after the fourth and fifth booster immunizations were used in the experiments.

Immunoprecipitation, Western blotting and analysis of receptor autophosphorylation

Receptor stimulation, cell lysis, immunoprecipitation and Western blotting followed previously published procedures (Joukov *et al.*, 1996). Immunoprecipitations of metabolically ^{35}S-labelled VEGF and VEGF-C from CM were carried out using mouse monoclonal anti-human VEGF neutralizing antibody (R&D Systems) and antiserum 882 or 905, respectively. VEGF and VEGF-C bound to antibodies were precipitated using protein G–Sepharose (Pharmacia) and protein A–Sepharose respectively. The peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig (DAKO), diluted 1:1000, and the ECL method (Amersham) were used to detect the polypeptide–antibody complexes on Western blots.

Generation of VEGF-C mutants

VEGF-C mutants were generated using the Altered Sites II *in vitro* Mutagenesis System (Promega). For this purpose, the *Bam*HI fragment of the VEGF-C cDNA from VEGF-C/pREP7 (Joukov *et al.*, 1996) was subcloned in antisense orientation into the pALTER-1 vector. To generate the VEGF-C point mutants, suitable oligonucleotides were synthesized and the mutagenesis procedure was carried out according to the manufacturer's instructions. To generate the N-His construct, an 84mer oligonucleotide was used to introduce the 6×His tag in place of Phe32 (between Ala31 and Glu33). The NT VEGF-C construct was obtained using an oligonucleotide encoding a stop codon instead of Lys214. The deletion mutants were produced by using a loop-out deletion strategy, as described in (Bergman *et al.*, 1995). A 65mer oligonucleotide was used to generate the ΔN VEGF-C construct, in which residues 32–102 of VEGF-C were deleted. In the second round mutagenesis procedure, ΔN VEGF-C and a 52mer oligonucleotide were used to introduce the 6×His tag followed by a stop codon and a *Not*I site immediately after Ile225 to generate ΔNΔC. The CT construct was generated on the basis of the N-His construct. Oligonucleotides (54 and 63mer) were used to introduce *Nco*I sites in the same reading frame, one at the 3′ end of the 6×His tag, and another one at the 5′ end of the sequence encoding the C-terminal part of VEGF-C (starting from Ser228). The resulting construct was subjected to *Nco*I digestion and ligation, giving rise to the construct encoding VEGF-C signal peptide followed by the 6×His tag and the C-terminal half of VEGF-C (additional proline and tryptophan residues were present between the 6×His tag and the C-terminus as a result of introduction of the *Nco*I site in the same reading frame). The mutant constructs in the pALTER vector were digested with *Hin*dIII and *Not*I, subcloned into *Hin*dIII–*Not*I-digested pREP7 and used to transfect 293-EBNA cells.

Strain GS115 of the yeast *P.pastoris* and the expression vector pIC9 (Invitrogen) were used according to the manufacturer's instructions to express ΔNΔC. The VEGF-C sequence was amplified by PCR with a sense primer encoding residues 103–108 and an antisense primer encoding residues 212–215, followed by a 6×His tag. *Eco*RI sites were introduced in the the 5′ and 3′ termini of the sense and antisense primers. The amplified fragment was fused in-frame to the yeast a-factor signal sequence in pIC9.

Purification and N-terminal sequence analysis of VEGF-C

Antibody 882 was employed to purify wt VEGF-C from 1.2 l of CM of transfected 293-EBNA cells by immunoaffinity chromatography. The IgG fraction isolated using protein A–Sepharose (Pharmacia) was covalently bound to CNBr-activated Sepharose CL-4B (Pharmacia) at a concentration of 5 mg of IgG/ml Sepharose resin (Harlow and Lane, 1988). N-His VEGF-C was isolated using Talon™ Metal Affinity Resin (Clontech). Yeast ΔNΔC VEGF-C was purified using Ni-NTA Superflow resin (QIAGEN). No contaminating proteins were detected when 2 μg of the yeast purified ΔNΔC was analysed by SDS–PAGE with subsequent

Coomassie R-250 or silver staining of the gel. The purified material was analysed by electrophoresis, Western blotting and N-terminal amino acid sequence analysis as described earlier (Joukov *et al.*, 1996). An additional sequence obtained during the analysis of the 29–30 kDa polypeptide, NH_2-AVVMTQTPAS-COOH, corresponded to the variable region of the Ig-κ chain, which was present in the purified material due to leakage from the affinity matrix.

Pulse–chase and dimerization studies

Metabolic labelling, immunoprecipitation and pulse–chase analysis of polypeptides were done essentially as described previously (Joukov *et al.*, 1996). To study the composition of the VEGF-C dimers, the labelled polypeptide bands electrophoresed under non-reducing conditions were cut out from the gel, soaked for 30 min in 1× gel-loading buffer containing 200 mM β-mercaptoethanol and subjected to a second SDS–PAGE under reducing conditions. Reduction of the disulfide bonds and alkylation of wt, ΔN and R102S VEGF-C were carried out by incubation of CM in the presence of 10 mM dithiothreitol for 2 h at room temperature with subsequent addition of 25 mM iodoacetamide and incubation for 20 min at room temperature.

For polypeptide cross-linking, DSS (Pierce) was added to the serum- and BSA-free CM at a concentration of 1 mM. After incubation for 1 h, the reaction was quenched by addition of 60 mM Tris–HCl (pH 7.4) and incubation was continued for 30 min. The cross-linked VEGF- and VEGF-C complexes were precipitated using anti-VEGF antibodies and antiserum 882, respectively.

Binding studies using receptor extracellular domains

R-3EC (a kind gift from Dr Katri Pajusola) or R-2EC (Cao *et al.*, 1996) were added to the labelled CM, supplemented with 0.5% BSA and 0.02% Tween-20. A similar amount of CM was used for immunoprecipitation with antiserum 882. After incubation for 2 h at room temperature, anti-VEGF-C antibodies and R-3EC protein were absorbed to protein A–Sepharose, and R-2EC was immunoprecipitated using anti-AP monoclonal antibodies (Genzyme) and protein G–Sepharose. The VEGF-C–receptor complexes were washed three times with ice-cold binding buffer (PBS, 0.5% BSA, 0.02% Tween-20) and twice with 20 mM Tris–HCl (pH 7.4). The same media were precipitated using anti-AP antibody and protein G–Sepharose or with protein A–Sepharose to control possible non-specific absorption.

Analysis of VEGF-C binding to cell surface receptors

Mouse recombinant VEGF164 (a kind gift from Dr Herbert Weich) and pure yeast ΔNΔC were labelled with ^{125}I using the Iodo-Gen reagent (Pierce), and purified by gel filtration on Sephadex G-15 (Pharmacia). The specific activities were 3.5×10^6 c.p.m./pmol and 3.0×10^6 c.p.m./pmol for VEGF and ΔNΔC, respectively. Transfected PAE cells grown on gelatinized 24-well plates (10^5 cells/well) were washed twice with 0.5 ml of binding buffer (Ham's F12 medium, 25 mM HEPES, pH 7.4, 0.1% BSA, 0.1% sodium azide) and incubated for 1.5 h at room temperature in 0.25 ml of binding buffer with increasing concentrations (in saturation analysis) or with a 100 pM concentration of the labelled factor and increasing concentrations of the non-labelled factor (in competition experiments). The cells were then placed on ice, washed three times with ice-cold PBS/0.1% BSA, lysed in 1 M NaOH and counted in a γ-counter. To estimate the non-specific values in saturation binding, the same determinations were done in the presence of unlabelled ΔNΔC.

In order to cross-link the iodinated factors to the cell surface receptors, cells grown on 10 cm gelatinized cell culture dishes were incubated for 1.5 h at room temperature in binding buffer, containing 400 pM of [^{125}I]VEGF or [^{125}I]ΔNΔC. After two washes with PBS, the incubation was continued in PBS containing 0.5 mM of [bis(sulfosuccinimidyl)suberate] (BS^3) (Pierce) for 30 min at room temperature. Then 50 mM Tris–HCl was added to quench the reaction, the cells were washed twice with Tris-buffered saline and lysed in RIPA buffer. The lysates were subjected to immunoprecipitation with VEGFR-2-specific antibodies, and the precipitated material was analysed by SDS–PAGE and autoradiography.

Analysis of VEGF-C biological activity

Mitogenic assays for VEGF-C were carried out by analysis of thymidine incorporation into BCE cells (Olofsson *et al.*, 1996a) and endothelial cell migration assays in the three-dimensional collagen gel as described in Joukov *et al.* (1996). Vascular permeability was determined by the Miles assay (Miles and Miles, 1952). Briefly, depilated guinea pigs were injected intracardially with 20 mg/kg of Evans Blue (Sigma) in 0.5 ml of isotonic saline. The analysed polypeptides were dissolved in PBS and injected intradermally in a volume of 0.1 ml into the back of guinea pigs. In some experiments, the analysed material was pre-treated with 15 μg/ml of anti-human VEGF neutralizing antibody (R&D systems). After 20 min, the animals were sacrificed under anaesthesia, skin at the injection sites was excised and the amount of extravasated dye was quantitated by elution of the dye with 4.0 ml of formamide for 4 days at 45°C and measuring the optical density of the eluate at 620 nm (Udaka *et al.*, 1970). Similar results were obtained in three separate experiments.

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